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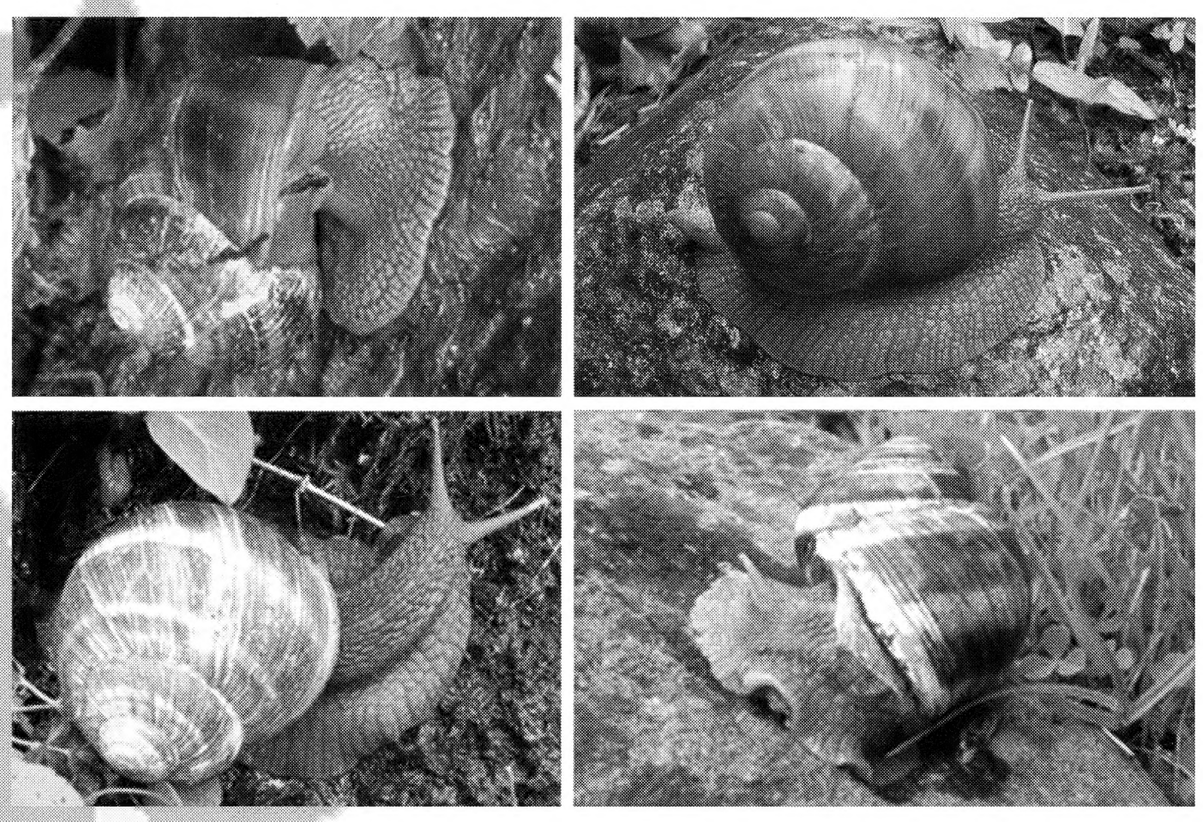

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Cover photo: Endemic Caucasian Helix (ECH) snails. Left: *Helix buchii* (top left, subadult; bottom left, adult); right: *Helix goderdziana* (top right, subadult; bottom, adult). Photograph courtesy of Mumladze *et al.* (pp. 225–234).

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Independent Papers

Research Notes

Corrigendum: Gilbertson *et al.* (AMB **31**: 57–64)

Latin gender change: *Cahuillus unifasciatus* (Willett, 1930)

In Gilbertson *et al.* (AMB **31**: 57–64), *Eremarionta rowelli unifasciata* (Willett, 1930) was reassigned to the genus *Cahuillus* Roth, 1996, and raised to full species status. The specific name of the new combination should have been emended to reflect the masculine gender of *Cahuillus* by changing its suffix to "-us." The purpose of this note is to correct the new binomen from *Cahuillus unifasciata* to *Cahuillus unifasciatus* (Willett, 1930). The DNA sequences associated with that publication have been updated in GenBank accordingly.

Amer. Malac. Bull. **31**(2): 213–224 (2013)

Are temperate land snails susceptible to climate change through reduced altitudinal ranges? A Pennsylvania example.

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Abstract: Distributions of some plants and animals have already shifted in recent years due to climate warming, and climate warming has potential to extirpate populations of taxa that cannot easily move or adapt to changes in temperatures and/or moisture. This study in Pennsylvania, U.S.A. focuses on whether snail populations currently confined to cooler habitats at higher elevations (elevations 700–978 m comprise only 2% of Pennsylvania's area) might decline or be eliminated if their ranges are reduced upward by climate warming. We examined whether some land snail species are limited to upper elevations in order to assess whether climate warming poses a threat to them. Sampling included 108 sites across Pennsylvania, comprising 12 localities at each of nine elevations from 100 to 900 m elev. Overall numbers of snail species and abundances decreased at greater elevations. Most individual species tended to occur throughout sampled elevations or occurred primarily at lower elevations, so the reduced altitudinal range aspect of climate warming might not threaten them. However, five species occurred significantly more often at greater elevations suggesting that their populations might decline if climate warming were to reduce their ranges upward. Four additional species including three native slugs showed non-significant trends to occur at higher elevations. These species might be monitored into the future.

Key words: elevation, climate warming, gastropods, population decline, threat

Climate change might affect biota directly, as through warming temperatures or changes in precipitation, or indirectly, by allowing new species into an area or causing a latitudinal shift in the range of the biota or its host organisms.

Climate warming has already altered the distributions of some plants and animals. Opossums (*Didelphis virginiana*) as assessed by road kills are shifting northward in Michigan (Myers *et al.* 2009). Distributions of high elevation plants in Europe are shifting upward so that at higher elevations, the more cold-adapted species decline and the more warm-adapted species increase (Gottfried *et al.* 2012). Climate change explains the upward range shifts of many small mammals in Yosemite National Park over the past century, resulting in range contractions of highland species unable to move higher than the mountaintops, and in upward expansions of upper limits of lowland species (Moritz *et al.* 2008).

Climate change has the potential to cause not just range shifts, but population extirpations in taxa that cannot easily move or adapt. Slow-moving snails might be susceptible to climate change; indeed, the Aldabra banded snail (*Rhachistia aldabrae* (von Martens, 1898)) gained the distinction of being the first documented species to become extinct due to modern climate change (Gerlach 2007).

Climate warming could threaten snails through elevational or latitudinal effects. Elevationally, populations currently confined to mountaintops (habitat islands) might perish if the climate warms and they cannot move higher to cooler conditions. Latitudinally, slow-moving land snails may be unable to disperse rapidly enough to stay within their shifting habitats.

Other climate warming threats not addressed in this paper could include movement into the area by other species (predators, parasites, diseases, or competitors), latitudinal shifts in range of the biota or its host organisms (*e.g.*, food or symbionts), and changes in precipitation, all of which are certainly expected from climate warming models, but are not sufficiently predictable for us to model here.

This study in the state of Pennsylvania, U.S.A., examines whether some land snail species are limited to upper elevations in order to assess whether climate warming might pose a threat to them. Sampling included 108 sites across Pennsylvania, comprising 12 localities at each 100 m increment from 100 to 900 m elev. As an additional test of the effect of elevation on snail distributions, we examined museum specimens having precise enough location data to assign reliable elevations.

MATERIALS AND METHODS

Pennsylvania topography

Pennsylvania elevations are not equally abundant and they are not distributed evenly throughout the state. Lowest

Presented as part of the Terrestrial Gastropod Symposium, Western Society of Malacologists, Santa Cruz, California, June 26, 2012.

(0–100 m) and highest (700–978 m) elevations are scarcest (Fig. 1), together making up only 5% of the area of the state, with less than 18 km^2 being greater than 900 m elevation. Highest elevations are distributed toward the southwest in the vicinity of Mt. Davis, Pennsylvania's high point, while lowest elevations are in the southeast in the vicinity of Philadelphia (Fig. 2).

From a climate perspective, moving poleward 1° latitude corresponds roughly to moving upward about 100 m elevation (Cogbill and White 1991). This adage predicts that climates within Pennsylvania will vary nearly 4 times as much by elevation as by latitude. Pennsylvania latitude spans 2.6° from 39.7° to 42.3° (equivalent to about 260 m elevation) while Pennsylvania elevation ranges from 0 m to 978 m (equivalent to about 9.8° latitude). Consequently, this project focuses primarily on elevation rather than latitude.

Sampling localities

We selected 108 localities (12 localities at each elevation in 100 m increments from 100 to 900 m elevation) throughout the state of Pennsylvania (Fig. 2). Forested, relatively undisturbed habitats were chosen by a knowledgeable worker from Pennsylvania Natural Heritage (PNH) at Western Pennsylvania Conservancy. He targeted areas with unusual plants and animals on the expectation that those localities might also have unusual snail faunas. After we had started sampling, we realized that some of these unusual organisms tracked by PNH occur in habitats such as acidic or dry sites that are unfavorable to snails. Nevertheless, we continued to use the sites selected by PNH without modification on the reasoning that understanding what influences snail distributions requires sampling the poor localities as well as the rich ones. We tried to spread sampling sites across Pennsylvania, attempting as best as we could to sample one third of the localities at each elevation from northern, middle, and southern latitudes. We were successful at spreading out the sample localities for 200–700 m elevations, but less successful at spreading the lowest and highest elevations because those elevations are concentrated in southern parts of the state. Choice of localities balanced the need for particular elevations, geographical proximity to access roads, minimally disturbed habitats, and land ownership.

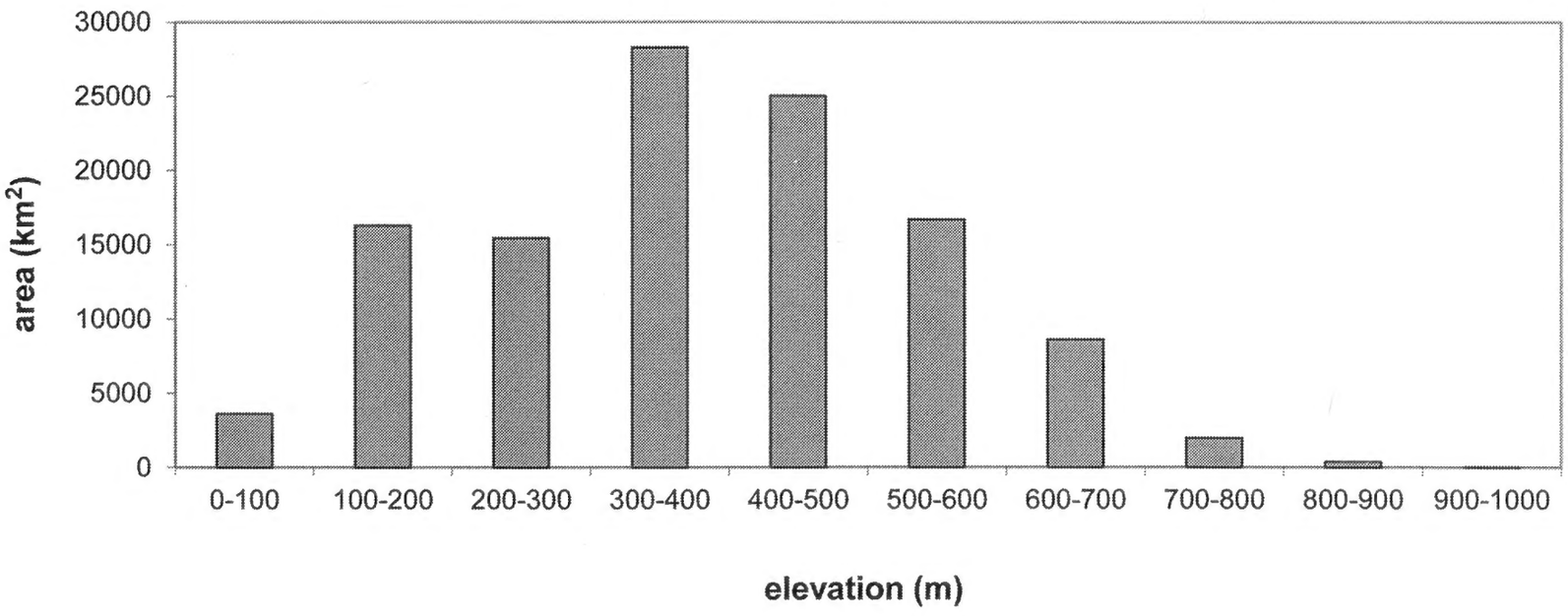

Figure 1. Elevational distribution of surface area in Pennsylvania. Higher elevations are scarcest. Areas covered by each 100 m span of elevation were calculated using GIS. With climate warming, taxa in the upper ranges of elevations will likely experience decreases in area occupied as they move to even higher elevations.

Survey methods

Field sampling

Sampling was conducted by the same two observers (T.A.P., M.E.P.) during the period of May to October, 2011. Within a 20 m diameter area at each site, we undertook 40 person minutes of visual search, and we collected 4.0 liters of leaf duff (decaying, fragmented leaves) from places that our experience has shown to be likely for snails, including microhabitats such as depressions in the earth or beside logs. The visual search targeted larger snails and slugs while the leaf duff samples were intended to recover smaller species (Emberton *et al.* 1996). Although Cameron and Pokryszko (2005) recommended sampling a minimum of 200 individuals in an area regardless of search effort, we wished to compare species richness and abundance across localities so we used equal sampling effort to be able to compare catch per unit effort (CPUE). We recovered more than 200 individuals in only 15 of 108 samples, which could limit our ability to make certain faunal comparisons, but we can still compare CPUE.

Since land snails average 3 mm (1/8 inch) diameter, adequately recovering the highly diverse minute species requires processing duff samples. The dried leaf duff was passed through nested sieves, and snails were picked out from all layers > 0.7 mm and some from the 0.5 mm layer (only rarely does the 0.5 mm layer contain species that were not found in larger fractions), using a microscope to examine smaller layers.

Most living specimens were collected and preserved as vouchers in alcohol, and dead-collected specimens were kept dry. We included live and dead collected individuals in our analyses because we were primarily interested in species occurrences and many species records were represented by empty shells only. We added together results from the visual search and leaf duff samples.

Museum data

We used museum data as an independent source of information to

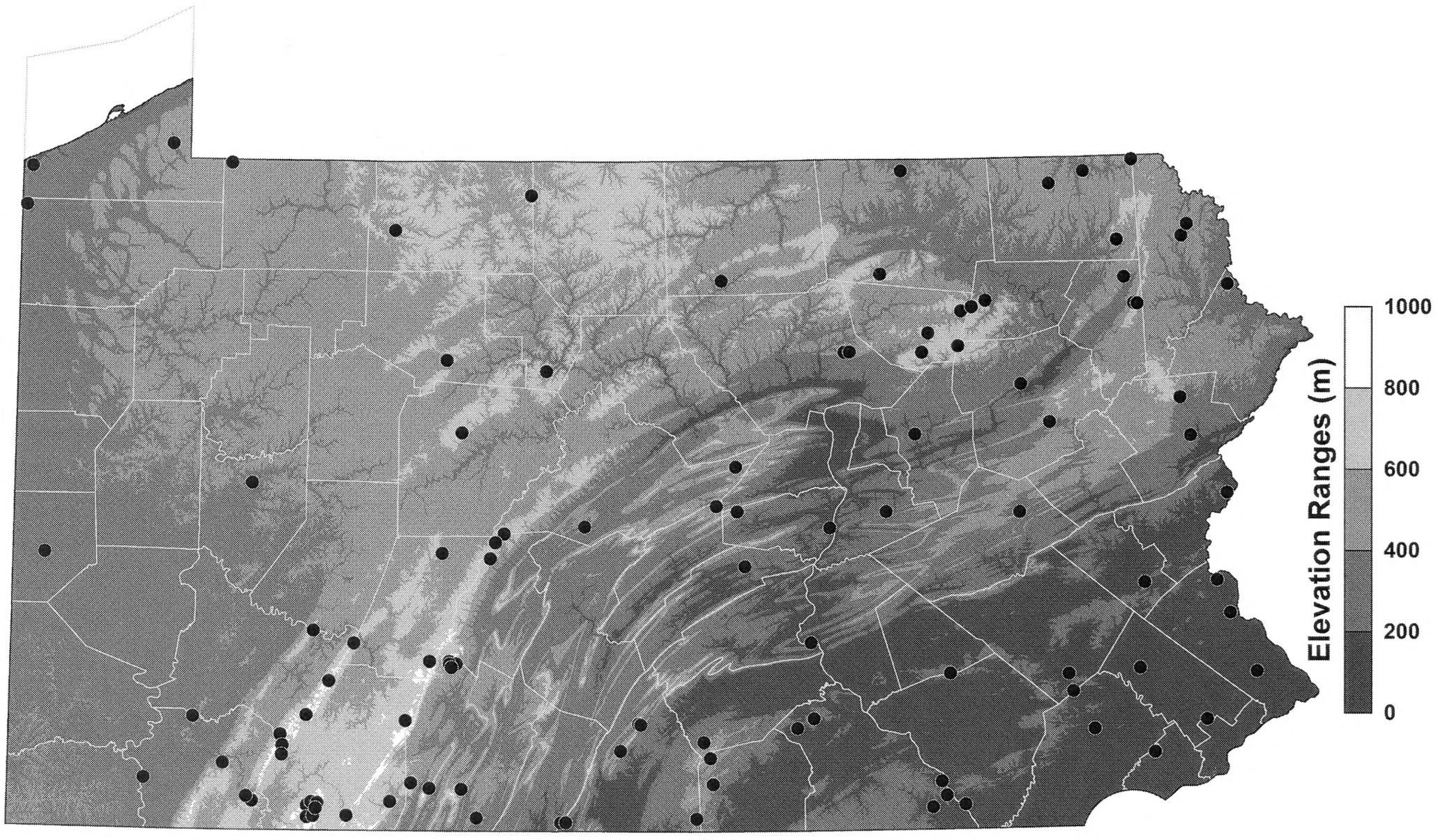

Figure 2. Samples were taken at 108 localities (circles) throughout Pennsylvania. Low-elevation sites (100 m) were necessarily concentrated in the southeastern part of the state, and high-elevation sites (800–900 m) were toward the southwest.

compare with the survey results and to increase sample size. Due to the nature of museum specimens, we can compare numbers of species at localities, but not number of individuals. We gathered 15,524 species locality records of terrestrial gastropods from eight major museums (American Museum of Natural History, Academy of Natural Sciences of Drexel University, Carnegie Museum of Natural History, Delaware Museum of Natural History, Field Museum, Florida Museum of Natural History, Museum of Comparative Zoology, and National Museum of Natural History). Of those records, 8403 had locality information sufficiently precise to be located within a 600 m radius, which we had arbitrarily decided would be precise enough to yield useful elevation information.

In contrast to our field samples, which were taken at 100 m elevation increments and had equal numbers of stations at each elevation, museum samples are from various elevations with variable numbers of samples from each elevation increment. For each species that occurred in our field sampling, we counted the number of museum occurrences in 100 m elevation ranges centered on even 100 m intervals (*e.g.*, 151–250 m, 251–350 m). The mid-range intervals had greater numbers of occurrences, probably reflecting that the mid range elevations are more extensive and have been sampled more. To standardize for elevation, we converted occurrences to percents by dividing each individual elevation range occurrence by the total occurrences of all included species for that elevation range (and multiplying by 100).

Identification

We identified snails and slugs with reference to identification guides (Pilsbry 1940, 1946, 1948, Burch 1962, Fairbanks 1986, 1990, Forsyth 2004, Dourson 2010, and Nekola and Coles 2010) and with reference to the research collection at Carnegie Museum of Natural History. Because of identification difficulty, we identified members of the family Succineidae to the level of family only and we treat them as a single taxon in this study, although observed variability suggests presence of more than one species. Adult Philomycidae were dissected to verify identity. Although numerous records of *Philomycus carolinianus* (Bosc, 1802) have been reported from Pennsylvania, we did not find any specimens of that taxon and we propose that this species may be mostly or entirely absent from the state and that most of the museum records for this taxon are probably misidentifications of other species of *Philomycus* Rafinesque, 1820. Some living juvenile Polygyridae were raised to adulthood to allow their identification. Juvenile specimens whose identities were uncertain were inferred to be the same species as adults in the same sample, or if no adults were present, they were identified to genus only.

We did not verify identities of most museum specimens. Results from our recently collected samples might differ from museum data if the latter are incorrectly identified. For example, some museum specimens that we would now call *Helicodiscus shimeki* (Hubricht, 1962) would have been identified as *Helicodiscus parallelus* (Say, 1817) before 1962, the year *H. shimeki* was recognized as a distinct species.

Data analysis

In order to examine overall faunal patterns, we examined species richness and abundances at each of the 108 localities across elevations. Regarding species, in order to determine whether species occurred more often at upper or lower elevations, we performed separate analyses of field sampling results and museum data results, using the number or percentage of occurrences of each species at each of the nine elevations. For all these examinations, we calculated a regression line and correlation coefficient to determine whether the slope of the line was significantly different from zero.

RESULTS

At the 108 sampling localities, we collected 11,007 individual specimens of 69 species, yielding 1137 species-occurrences. Overall, snails in this study showed greater species richness (Fig. 3) and abundance (Fig. 4) at lower elevations. However, number of individuals tends to correlate positively with number of species (S) so our finding of fewer species at higher elevations could be an artifact of our finding fewer individuals there. To examine this possibility, we correlated low (100–300 m), medium (400–600 m), and high (700–900 m) elevation S with number of individuals and found a lower slope (although not significantly lower) for the high elevation trajectory (for the equation y = a * Ln(x) + b, slopes [a] of low, medium, and high elevations were 4.3917, 3.9015, and 2.8999, respectively, $p > 0.05$). This finding is consistent with the observation that higher elevations had fewer species. Furthermore, the Chao estimator (which estimates the number of missed species; Cameron and Pokryszko 2005) plus observed S at each locality (omitting samples with fewer than 50 individuals) tended to be lower for the upper elevations (for low, medium, and high elevations, mean Chao + S was 16.7, 20.2, and 15.1, respectively; t-test between medium and high $p = 0.132$). Again, this finding is consistent with the observation that higher elevations might actually have had fewer species.

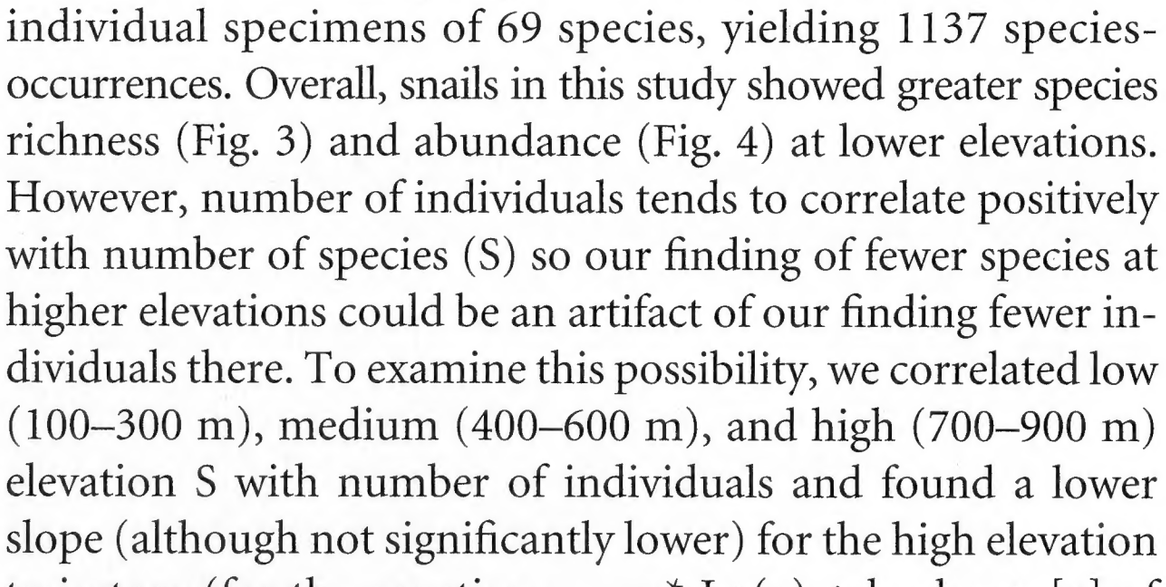

Figure 3. Species richness across elevations. Fewer species were found at higher elevations ($r = 0.6570$, d.f. = 8, $p < 0.05$). Error bars are standard deviation.

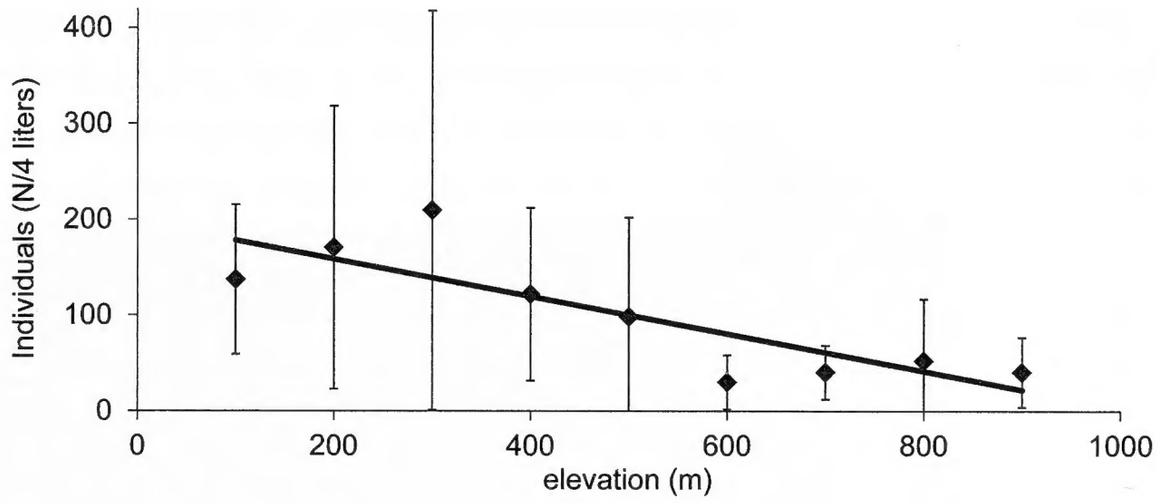

Figure 4. Number of individuals per sample (in 4 liters of leaf duff and 40 person minutes of visual search). Fewer individuals were found at higher elevations ($r = 0.8316$, d.f. = 8, $p < 0.005$). Error bars are standard deviation.

For the 69 individual species, occurrences of five species showed significant positive slopes (greater occurrences at higher elevations) and 32 showed significant negative slopes in at least one of the field sampling and museum data results (Table 1). The slopes of 29 species did not differ from zero slope, and three had insufficient data for analysis. Numbers of occurrences at the different elevations are shown for field sampling in appendix 1 and museum data in appendix 2. Slopes of the regressions for individual species are shown in Table 1.

Although we made 109 comparisons, we did not apply corrections to the *p*-values for multiple comparisons and we recognize that by chance we can expect about 5% to appear significant at $p < 0.05$. Consequently, about 5 of the 37 significant slopes could have appeared significant by chance, but there is no way to know which ones are spurious. The fact that we have similar trends in the data from field collections and from museum data strengthens the veracity of these trends. Furthermore, as stated above, finding significantly greater occurrences for five species at higher elevations runs against the trend we would expect given the bias against finding species at higher elevations due to smaller numbers of individuals.

Five species had significant positive slopes, two in both field sampling and museum data (*Mesomphix perlaevis* Pilsbry, 1900) and *Striatura ferrea* (E. S. Morse, 1864)), one in field sampling data only (*Helicodiscus shimeki* (Hubricht, 1962)), one in museum data only (*Striatura milium* (E. S. Morse, 1859)), and one significant in field sampling data

Table 1. Slope of regression of occurrences (Occ) of 69 Pennsylvania land gastropods found across 9 elevations. Negative slopes indicate taxa that are scarcer at higher elevations; positive slopes are those with more occurrences at higher elevations. Results are given separately for field sampling and museum data. Correlation coefficient (*r*) and p-value (*p*, not corrected for multiple comparisons) are shown for species having at least 6 occurrences. Slopes in bold are significantly different from a zero slope. No slope is shown for p-values greater than 0.2.

	Field Sampling Data				Museum Data			
	Occ	**slope**	***r***	***p***	**Occ**	**slope**	***r***	***p***
Allogona profunda (Say, 1821)	1				68	-0.0014	0.315	0.1
Anguispira alternata (Say, 1816)	6		0.046	>.5	201	**-0.0061**	0.822	0.001
Appalachina sayana (Pilsbry, 1906)	4				23		0.190	>.5
Arion intermedius (Normand, 1852)	7	**-0.0043**	0.692	<.05	20	**-0.0009**	0.627	0.01
Arion subfuscus (Draparnaud, 1805)	37	-0.0045	0.509	>.1	138		0.001	>.5
Carychium exile I. Lea, 1842	8	**-0.0025**	0.650	<.05	123	**-0.0021**	0.537	0.02
Cochlicopa lubrica (Müller, 1774)	2				139	-0.0033	0.322	0.1
Cochlicopa lubricella (Porro, 1838)	2				30	**-0.0009**	0.789	0.001
Cochlicopa morseana Doherty, 1878	2				31		0.053	>.5
Columella simplex (Gould, 1840)	15	**-0.0027**	0.653	<.05	118	**-0.0019**	0.426	0.05
Deroceras laeve (Müller, 1774)	1				20	-0.0006	0.321	0.1
Deroceras reticulatum (Müller, 1774)	1				51	**-0.0015**	0.433	0.05
Discus catskillensis (Pilsbry, 1896)	10		0.000	>.5	26		0.129	>.5
Discus patulus (Deshayes, 1830)	3				76	**-0.0016**	0.433	0.05
Euchemotrema fraternum (Say, 1824)	12	**-0.0033**	0.745	<.02	80		0.038	>.5
Euconulus dentatus (Sterki, 1893)	1							
Euconulus polygyratus (Pilsbry, 1899)	21	**-0.0073**	0.820	<.005	187	**-0.0041**	0.735	0.002
Gastrocopta armifera (Say, 1821)	2				59	**-0.0026**	0.676	0.005
Gastrocopta contracta (Say, 1822)	5				92	**-0.0040**	0.600	0.01
Gastrocopta pentodon (Say, 1822)	28	**-0.0045**	0.699	<.05	157	-0.0026	0.384	0.1
Glyphyalinia indentata (Say, 1823)	66	**-0.0075**	0.678	<.05	428		0.021	>.5
Glyphyalinia rhoadsi (Pilsbry, 1899)	32		0.333	>.2	119		0.040	>.5
Glyphyalinia wheatleyi (Bland, 1883)	22	-0.0045	0.596	>.05	160	**-0.0022**	0.458	0.05
Guppya sterki (Dall, 1888)	14	-0.0038	0.523	>.1	59		0.103	>.5
Haplotrema concavum (Say, 1821)	7		0.329	>.2	161	**-0.0033**	0.597	0.01
Hawaiia minuscula (A. Binney, 1841)	4				114	**-0.0032**	0.835	0.001
Helicodiscus parallelus (Say, 1817)	26	**-0.0062**	0.775	<.01	169		0.157	>.5
Helicodiscus shimeki (Hubricht, 1962)	17	**0.0082**	0.834	<.005	45		0.146	>.5
Lucilla singleyana (Pilsbry, 1889)	7	**-0.0028**	0.710	<.05	33	**-0.0012**	0.538	0.02
Megapallifera mutabilis (Hubricht, 1951)	10	**-0.0038**	0.683	<.05	10		0.280	>.5
Mesodon thyroidus (Say, 1816)	12	**-0.0045**	0.779	<.01	261	**-0.0056**	0.620	0.01
Mesodon zaletus (A. Binney, 1837)	5				66		0.245	>.5
Mesomphix cupreus (Rafinesque, 1831)	10		0.062	>.5	168	-0.0025	0.349	0.1
Mesomphix inornatus (Say, 1821)	35	0.0067	0.622	>.05	235		0.000	>.5
Mesomphix perlaevis (Pilsbry, 1900)	10	**0.0027**	0.693	<.05	102	**0.0096**	0.462	0.05
Neohelix albolabris (Say, 1817)	14		0.431	>.2	220	-0.0023	0.336	0.1
Neohelix dentifera (A. Binney, 1837)	21	**0.0043**	0.634	<.05	83	0.0165	0.324	0.1
Pallifera dorsalis (A. Binney, 1842)	31	0.0033	0.548	>.1	28		0.001	>.5
Pallifera fosteri F.C. Baker, 1939	1				6		0.079	>.5
Pallifera secreta (Cockerell, 1900)	3							
Pallifera varia Hubricht, 1953	4							
Paravitrea multidentata (A. Binney, 1840)	12		0.082	>.5	104		0.153	>.5
Philomycus flexuolaris Rafinesque, 1820	43	0.0058	0.576	>.05	19		0.004	>.5
Philomycus togatus (Gould, 1841)	35	0.0037	0.569	>.05	16		0.216	>.5
Punctum minutissimum (I. Lea, 1841)	64	-0.0052	0.561	>.05	476		0.001	>.5
Punctum vitreum (H.B. Baker, 1930)	2				64	**-0.0019**	0.509	0.05
Stenotrema barbatum (G.H. Clapp, 1904)	1				14		0.019	>.5
Stenotrema hirsutum (Say, 1817)	10	**-0.0035**	0.822	<.005	133	**-0.0035**	0.847	0.001

Table 1. (Continued)

	Field Sampling Data				Museum Data			
	Occ	**slope**	***r***	***p***	**Occ**	**slope**	***r***	***p***
Striatura exigua (Stimpson, 1850)	11		0.421	>.2	37		0.008	>.5
Striatura ferrea E.S. Morse, 1864	82	**0.0097**	0.916	<.001	321	**0.0302**	0.638	0.01
Striatura meridionalis (Pilsbry, and Ferriss, 1906)	13	-0.0040	0.583	>.05	94		0.054	>.5
Striatura milium (E.S. Morse, 1859)	53		0.085	>.5	195	**0.0096**	0.434	0.05
Strobilops aeneus Pilsbry, 1926	2				34	**-0.0012**	0.755	0.002
Strobilops labyrinthicus (Say, 1817)	4				21	**-0.0010**	0.593	0.01
Strobilops texasianus Pilsbry and Ferriss, 1906	5				4		0.028	>.5
Succineidae	17	-0.0042	0.581	>.05	195		0.133	>.5
Triodopsis juxtidens (Pilsbry, 1894)	1				52	**-0.0017**	0.822	0.001
Triodopsis tridentata (Say, 1816)	28		0.254	>.2	292		0.001	>.5
Triodopsis vulgata Pilsbry, 1940	6	**-0.0018**	0.710	<.05	13		0.169	>.5
Vallonia costata (Müller, 1774)	1				41	**-0.0022**	0.560	0.02
Ventridens intertextus (A. Binney, 1841)	20		0.030	>.5	113		0.089	>.5
Ventridens ligera (Say, 1821)	26		0.022	>.5	430		0.001	>.5
Ventridens suppressus (Say, 1829)	16	**-0.0062**	0.825	<.005	121	**-0.0044**	0.821	0.001
Ventridens virginicus (Vanatta, 1936)	2				18	**-0.0005**	0.690	0.005
Vertigo bollesiana (E.S. Morse, 1865)	1				12		0.064	>.5
Vertigo gouldii (A. Binney, 1843)	7		0.219	>.5	38	**-0.0013**	0.689	0.005
Xolotrema denotata (Férussac, 1821)	8	-0.0020	0.520	>.1	83		0.272	>.5
Zonitoides arboreus (Say, 1816)	77	**-0.0060**	0.701	<.05	403		0.049	>.5
Zonitoides nitidus (Müller, 1774)	1				56	**-0.0010**	0.422	0.05

and non-significant positive in museum data (*Neohelix dentifera* (A. Binney, 1837)). It is interesting to note that of four species having non-significant positive slopes (*Mesomphix inornatus* (Say, 1821), *Pallifera dorsalis* (A. Binney, 1842), *Philomycus flexuolaris* (Rafinesque, 1820), and *Philomycus togatus* (Gould, 1841)) three are native slugs. Examples from the field sampling data of species showing greater occurrences at higher and lower elevations, or throughout, are shown in Fig. 5.

DISCUSSION

We found fewer species and lower numbers of land gastropods at higher elevations. This result is in accord with the findings of many land snail surveys that species richness generally decreases with elevation in both temperate and tropical settings (Cameron 1978, Tattersfield *et al.* 2001, Aubry *et al.* 2005, Liew *et al.* 2010, Presley *et al.* 2011, but for counter examples see Emberton 1997 and Emberton *et al.* 1997). Our finding of fewer species at higher elevations is likely to be at least partly a result of our finding fewer individuals there. However, it remains inconclusive whether higher elevations actually had as many species as lower elevations but our sampling didn't find them all, or whether higher elevations actually had fewer species. This uncertainty actually strengthens our finding that some species occur more frequently at higher elevations because this result appeared despite a bias tending to diminish the chance of finding any species at higher elevations.

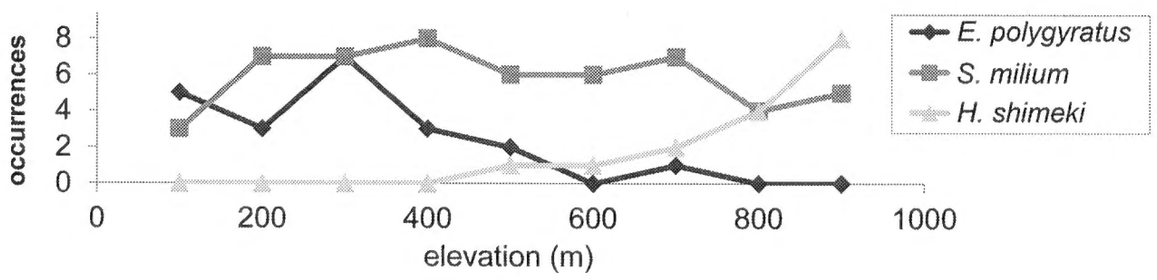

Figure 5. Examples of snail species from the field sampling that were more commonly found at lower elevations (*Euconulus polygyratus*) (Pilsbry, 1899), found at upper elevations (*Helicodiscus shimeki*), or found throughout the elevation range (*Striatura milium*).

Regarding individual species, we found that most tended to occur more frequently and at greater abundances at lower elevations or throughout sampled elevations so any reduced-range-upward aspect of climate warming might not threaten those species in the foreseeable future. However, five species significantly and four non-significantly occurred more frequently at higher elevations, suggesting that their populations within Pennsylvania could decline if climate warming were to reduce their ranges upward. Since elevations above 700 m are quite scarce in Pennsylvania (2.0% of land area), if medium elevation species are

shifted upward, they would be forced into much smaller areas and their populations could decline markedly. All of these species are relatively common in Pennsylvania at the present time, but they should be monitored into the future to verify whether climate warming is affecting them negatively.

Suitable baseline data on distributions are lacking for land gastropods in most parts of the world, including Pennsylvania, but we can refer to museum data for historical records. As an example, the snail *Anguispira alternata* (Say, 1816) is relatively large (diameter about 18–24 mm; Pilsbry 1948) and at least historically it has been relatively common, which probably explains the relatively large number of museum specimens for this species. In recent decades, *A. alternata* has not been found in half of the counties where it was previously known despite considerable search effort (Fig. 6). We have examined potential sources of bias, such as collecting bias in which common species may be collected abundantly at first. Amount of search effort and these potential biases do not seem to be responsible for the apparent decline in distribution. Many of the counties from which it has disappeared are in southeastern Pennsylvania, which has the lowest elevations in the state (Fig. 6). There has been an upward shift of the elevation range of *A. alternata* in Pennsylvania; considering the 199 localities for this species that had precise enough localities to estimate reliable elevations, the mean elevation before 1960 was 259 m ($n = 127$, range 18–676 m), while the mean elevation since 1960 was 352 m ($n = 72$, range 88–656 m; t-test, $p = 0.00001$). This upward elevation shift is consistent with predictions of global warming and mirrors the trend of climate change seen in Pennsylvania since 1960 (UCS 2008). Although the distribution of *A. alternata* includes areas to the south of Pennsylvania as far as northern Alabama (Hubricht 1985), those southern areas are largely areas of higher elevation. If the climate change models predicted for Pennsylvania are accurate, and if temperature is a major influence on the distribution of *A. alternata*, then we predict the future distribution of this species will be limited to the higher elevation north central region of Pennsylvania by the end of this century, if it is not extirpated.

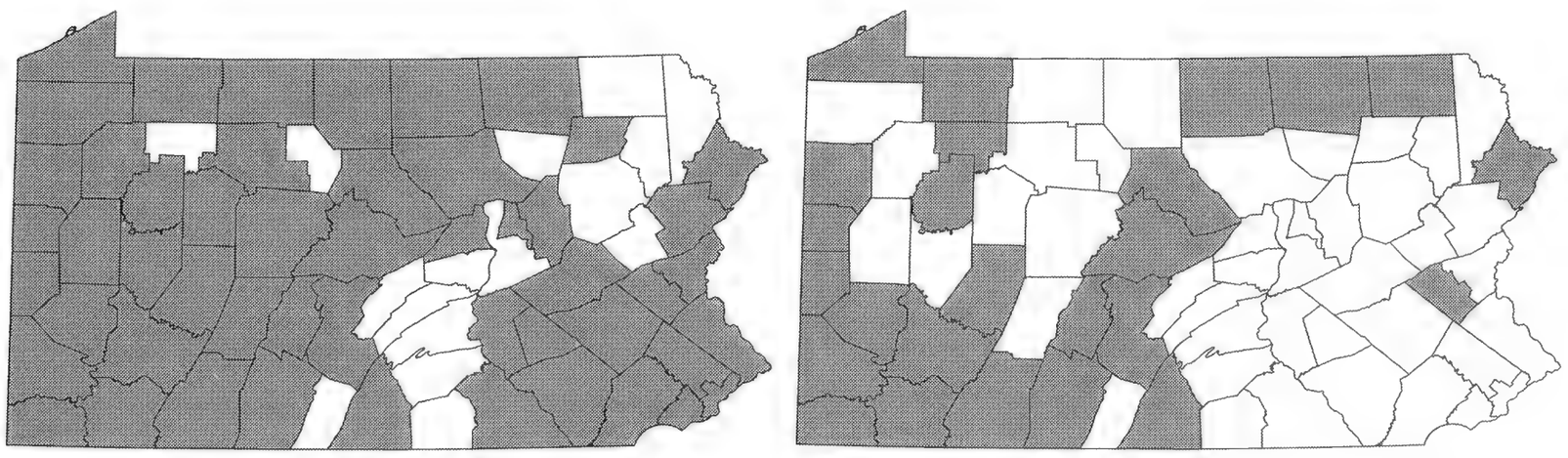

Figure 6. *Anguispira alternata* county occurrences reported from Pennsylvania before 1960 (left) and after 1960 (right). Lowest elevations are in the southeastern part of the state and the disappearance of *A. alternata* from those counties suggests an upward shift of its lowest elevation limit, a shift that is consistent with climate warming.

While we focused on climate warming, climate change is expected to impact mollusks in other ways. Another direct effect of climate change that will likely affect land snails is changes in rainfall. Some indirect effects are expected due to shifting habitats and the addition of new species to communities.

Changes in rainfall are a great concern because snails need moisture. The first species extinction attributed to climate change is a land snail whose extinction is attributed to reduced rainfall that increased mortality of juveniles (Gerlach 2007). Climate change related alterations in precipitation will likely affect land snails in Pennsylvania.

As habitats shift pole-ward due to climate warming, we can expect snails to be affected more than many other organisms due to their limited dispersal ability. Furthermore, human modifications such as fragmentation of habitats would make movement in response to climate change more difficult. Because of their small size and low motility, many snail species face serious barriers to dispersal, hindering them from migrating with their shifting habitats. Land snails are one of the most imperiled groups of organisms (Lydeard *et al.* 2004), and those on islands seem especially vulnerable (Bauman 1996, Cowie and Robinson 2003). Many land snail species effectively occupy island habitats (*e.g.*, mountaintops, outcrops, or microhabitats with a narrow moisture range) and are isolated by even relatively small fragmenting features that may be inconsequential to larger, more motile animals (Baur and Baur 1990). Many living components of habitats might be able to leap gaps or cross uninhabitable areas that would be more difficult for snails to cross. Conservation of land snails against climate change might require interventions such as assisted migration (Örstan 2009).

Endemism (limited range) can be much more common among highland species of land snails than among lowland species (Emberton *et al.* 1997, Liew *et al.* 2010, Dirnböck *et al.* 2011, but for a counter example see Emberton 1997), such that high-elevation taxa already have a greater risk of extinction prior to any habitat change.

Evidence from cave deposits and modern distributions suggests that snails might disperse more slowly than their habitats. In a cave on the Gaspé Peninsula in Quebec (Pearce *et al.* 2010), four land snail species (*Discus whitneyi* (Newcomb, 1864), *Zonitoides arboreus* (Say, 1816), *Anguispira alternata*, and

Neohelix albolabris (Say, 1817)) were present at the bottom of the cave deposits along with bones of three tundra mammals (collared lemming, yellow-cheeked vole, Arctic hare), implying that those snails occupied tundra conditions in the past (estimated 7300–9000 BP). Of those four snail species, the northernmost modern occurrences of the two smaller species (whose passive dispersal might be faster) are far north in the northern part of the boreal forest just south of tundra, but the northernmost modern occurrences of the two larger species (which might disperse more slowly) are found much farther south, at the southern edge of the boreal forest. This circumstantial evidence suggests that the larger snails have not dispersed northward as far or rapidly as their habitat has.

The indirect effect of other species moving in and out of the community can be expected to affect snails. Most land snails appear to be generalist herbivores, so particular food species becoming scarce might not have much effect on snail species. On the other hand, if climate change allows new species to move into the community, the new arrivals might affect snails if they are predators, competitors, parasites, or diseases. Interestingly, three species of native land slugs (*Pallifera dorsalis* (A. Binney, 1842), *Philomycus flexuolaris, and Philomycus togatus*), showed non-significant positive trends to occur at higher elevations, while the introduced slug *Arion intermedius* (Normand, 1852) showed significantly and *A. subfuscus* (Draparnaud, 1805) non-significantly more occurrences at lower elevations. In this case, the slug introductions are due to human agency and not related to climate warming. All three of the native species also occur south of Pennsylvania (Hubricht 1985), but those southern occurrences are largely areas of higher elevation. Although no evidence exists for competition between the native and non-native species (Paustian 2010, Paustian and Barbosa 2012), whether the native slugs have been displaced upward by the non-native slugs or lowland habitat alteration or whether they prefer the cooler climates is unknown, but warming climate will likely reduce the ranges of these native slugs in Pennsylvania.

Assessing the elevation distribution of rare species is difficult because sample sizes are usually too small to draw meaningful conclusions. Some species of land snails are uncommon in Pennsylvania and most of those (*e.g.*, *Carychium nannodes* G. H. Clapp, 1905, *Webbhelix multilineata* (Say, 1821), *Hendersonia occulta* (Say, 1831), *Glyphyalinia raderi* (Dall, 1898), and *Gastrodonta interna* (Say, 1822)) were not encountered in our field sampling. Some of the rare species are associated with particular habitats, such as high quality limestone areas (Pearce 2008), but whether they are additionally threatened by climate warming remains to be determined.

In conclusion, although we found the overall numbers of species and abundances of land gastropods to decrease at higher elevations, the five snail species (significant) and one snail and three slug species (non-significant trend) that occur more at higher elevations will likely suffer marked population declines as the climate warms.

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Appendix 1. Species occurrences (N-occ) at various elevations (m). Field sampling data.

	N-occ	100	200	300	400	500	600	700	800	900
Allogona profunda	1					1				
Anguispira alternata	6			1	1	3		1		
Appalachina sayana	4	1		1		1		1		
Arion intermedius	7	5	2							
Arion subfuscus	37	9	2	4	4	5	4	4	5	
Carychium exile	8	2	3		1	1			1	
Cochlicopa lubrica	2		1	1						
Cochlicopa lubricella	2		1		1					
Cochlicopa morseana	2			2						
Columella simplex	15	3	2	3	2	2		1		2
Deroceras laeve	1	1								
Deroceras reticulatum	1			1						
Discus catskillensis	10		1	1	3	2	1		1	1
Discus patulus	3				1	1			1	
Euchemotrema fraternum	12	2	3	3	2			1	1	
Euconulus dentatus	1			1						
Euconulus polygyratus	21	5	3	7	3	2		1		
Gastrocopta armifera	2		1	1						
Gastrocopta contracta	5	1	2	2						
Gastrocopta pentodon	28	5	2	6	5	3	2	2	2	1
Glyphyalinia indentata	66	7	10	11	11	9	4	4	6	4
Glyphyalinia rhoadsi	32	2	1	5	5	4	5	2	4	4
Glyphyalinia wheatleyi	22	2	6	5	2	3		1	3	
Guppya sterki	14		5	2	4	3				
Haplotrema concavum	7		2	2		2			1	
Hawaiia minuscula	4		1	2					1	
Helicodiscus parallelus	26	5	7	3	2	3	1	1	3	1
Helicodiscus shimeki	17	1				1	1	2	4	8
Lucilla singleyana	7	3	2	1					1	
Megapallifera mutabilis	10	3	2	4					1	
Mesodon thyroidus	12	4	4	1		1	1	1		
Mesodon zaletus	5			1	1	1			1	1
Mesomphix cupreus	10			2	2	4		2		
Mesomphix inornatus	35		1	3	6	7	1	3	8	6
Mesomphix perlaevis	10				1	2	1	3	2	1
Neohelix albolabris	14	1	2	4	1	2	4			
Neohelix dentifera	21		1	3	2	3	2		5	5
Pallifera dorsalis	31	2	2	3	3	4	6	1	5	5
Pallifera fosteri	1				1					
Pallifera secreta	3				1					
Pallifera varia	4				1	3				
Paravitrea multidentata	12			2	4	4		1	1	
Philomycus flexuolaris	43		2	3	9	6	6	7	4	6
Philomycus togatus	35	2	2	5	5	3	2	4	7	5
Punctum minutissimum	64	10	8	10	8	6	2	8	5	7
Punctum vitreum	2	1		1						
Stenotrema barbatum	1		1							
Stenotrema hirsutum	10	2	2	3	1	2				
Striatura exigua	11		2	3	3	2			1	
Striatura ferrea	82	4	5	8	9	11	11	11	11	12
Striatura meridionalis	13	1	6	2	1	2			1	
Striatura milium	53	3	7	7	8	6	6	7	4	5
Strobilops aeneus	2			2						

Appendix 1. (Continued)

	N-occ	100	200	300	400	500	600	700	800	900
Strobilops labyrinthicus	4	1		1	1	1				
Strobilops texasianus	5	3	1	1						
Succineidae	17	5	5	1	1			3	1	1
Triodopsis juxtidens	1					1				
Triodopsis tridentata	28	2	2	3	4	6	2	1	3	5
Triodopsis vulgata	6	1	1	2	1		1			
Vallonia costata	1									1
Ventridens intertextus	20		4	1	4	4	2	1	3	1
Ventridens ligera	26	5	4	3	1	2		2	2	7
Ventridens suppressus	16	4	6	2	2		1	1		
Ventridens virginicus	2			2						
Vertigo bollesiana	1					1				
Vertigo gouldii	7		1	2	2		1			1
Xolotrema denotata	8	1	1	2	1	3				
Zonitoides arboreus	77	11	11	10	7	11	8	8	4	7
Zonitoides nitidus	1		1							

Appendix 2. Species occurrences (N-occ) at various elevations. Museum data (% occurrence in each elevation range).

Species	N-occ	51-150	151-250	251-350	351-450	451-550	551-650	651-750	751-850	851-950
Allogona profunda	68		1.79	1.50	0.34	0.14				
Anguispira alternata	201	5.95	3.35	2.86	2.19	1.41	1.31	2.02		
Appalachina sayana	23		0.45	0.27	0.34	0.42	0.33			
Arion intermedius	20	0.54	0.89	0.27	0.06	0.14				
Arion subfuscus	138	1.62	1.56	0.90	2.69	0.85	0.98	3.03	3.13	
Carychium exile	123	0.81	2.01	2.19	1.40	0.99	0.66	1.01		
Cochlicopa lubrica	139	4.59	1.79	2.33	1.07	0.42	0.33		3.13	
Cochlicopa lubricella	30	0.81	0.33	0.53	0.28	0.28	0.33			
Cochlicopa morseana	31	0.27		0.60	0.45	0.14	0.98			
Columella simplex	118	1.08	1.00	1.93	1.96	0.99	1.64			
Deroceras laeve	20	0.81	0.11	0.33	0.22		0.66			
Deroceras reticulatum	51	1.89	0.67	0.83	0.34		0.66	1.01		
Discus catskillensis	26		0.45	0.33	0.34	0.42	0.66		3.13	
Discus patulus	76	0.27	1.90	1.30	0.84	0.28	0.66			
Euchemotrema fraternum	80		0.45	1.33	1.63	0.42	0.66		3.13	
Euconulus dentatus	0									
Euconulus polygyratus	187	1.89	3.35	2.96	2.53	1.41	1.64			
Gastrocopta armifera	59	2.70	0.89	0.90	0.51	0.28				
Gastrocopta contracta	92	4.32	1.00	1.46	0.51	0.42				
Gastrocopta pentodon	157	2.43	1.45	1.76	2.81	2.55	2.95	1.01		
Glyphyalinia indentata	428	3.78	4.69	5.15	6.79	7.64	6.23	11.11	9.38	
Glyphyalinia rhoadsi	119	1.62	1.56	1.33	1.23	2.55	3.61	3.03		
Glyphyalinia wheatleyi	160	1.08	1.56	2.76	2.24	1.27	1.31	1.01		
Guppya sterki	59		0.22	1.10	1.12	0.14	0.66			
Haplotrema concavum	161	1.08	2.68	2.93	1.80	0.85				
Hawaiia minuscula	114	2.43	1.34	1.76	1.68	0.71				
Helicodiscus parallelus	169	4.32	2.23	2.03	2.30	0.99	0.66	1.01	3.13	12.50
Helicodiscus shimeki	45	0.27		0.30	0.51	2.97	0.98		6.25	
Lucilla singleyana	33	1.35	0.11	0.47	0.56	0.14				
Megapallifera mutabilis	10	0.54		0.07	0.22	0.14	0.33			

Appendix 2. (Continued)

Species	N-occ	51-150	151-250	251-350	351-450	451-550	551-650	651-750	751-850	851-950
Mesodon thyroidus	261	5.14	4.69	3.26	3.98	1.13	0.33	1.01	3.13	0.00
Mesodon zaletus	66	0.27	1.12	0.80	1.01	1.56	0.33			12.50
Mesomphix cupreus	168	0.81	1.90	2.86	2.47	1.56	2.30			
Mesomphix inornatus	235		3.57	3.03	2.97	5.23	5.90	2.02	3.13	
Mesomphix perlaevis	102	0.27	0.33	0.86	1.80	4.53	1.64	1.01	3.13	12.50
Neohelix albolabris	220	2.70	3.79	3.03	2.86	2.55	1.64	3.03	3.13	
Neohelix dentifera	83		1.79	0.53	1.07	2.83	2.62	2.02		25.00
Pallifera dorsalis	28		0.11	0.20	0.34	1.70	0.66			
Pallifera fosteri	6	0.27		0.07	0.00	0.28	0.33			
Pallifera secreta	0									
Pallifera varia	0									
Paravitrea multidentata	104	0.27	1.00	1.50	1.96	0.99	1.97			
Philomycus flexuolaris	19	0.27		0.03	0.22	1.56	0.66			
Philomycus togatus	16	0.54		0.13	0.28	0.57	0.33			
Punctum minutissimum	476	4.86	4.46	6.05	7.30	8.35	7.54	14.14	6.25	
Punctum vitreum	64	2.16	0.45	1.10	0.67	0.28	1.31			
Stenotrema barbatum	14		0.22	0.27	0.11	0.00	0.66			
Stenotrema hirsutum	133	2.70	1.56	2.36	1.57	0.57	0.98			
Striatura exigua	37		0.67	0.37	0.67	0.71	0.66	1.01		
Striatura ferrea	321	0.81	1.79	2.33	5.50	9.90	9.84	23.23	31.25	12.50
Striatura meridionalis	94		1.23	1.26	1.52	1.27	1.97	1.01		
Striatura milium	195	0.27	1.23	2.10	3.65	3.68	5.90	7.07		12.50
Strobilops aeneus	34	1.08	0.56	0.60	0.22					
Strobilops labyrinthicus	21	1.08	0.22	0.27	0.28	0.14				
Strobilops texasianus	4			0.10		0.14				
Succineidae	195	4.59	4.46	2.10	2.19	1.70	0.66	1.01	6.25	
Triodopsis juxtidens	52	1.35	1.00	0.76	0.51					
Triodopsis tridentata	292	1.62	4.58	4.12	3.09	4.38	5.90	4.04	6.25	
Triodopsis vulgata	13		0.67	0.17	0.06		0.33			
Vallonia costata	41	2.43	0.89	0.43	0.06					
Ventridens intertextus	113	0.54	0.67	1.20	2.64	2.12	2.30			
Ventridens ligera	430	6.49	8.26	5.95	3.76	6.36	4.26	5.05		12.50
Ventridens suppressus	121	3.78	2.34	1.36	1.35	0.57				
Ventridens virginicus	18	0.27	0.33	0.20	0.39	0.14				
Vertigo bollesiana	12	0.27	0.11	0.07	0.28	0.14	0.66			
Vertigo gouldii	38	1.35	0.33	0.57	0.51	0.28	0.33			
Xolotrema denotata	83	0.54	1.90	1.16	0.90	0.99	1.64	1.01		
Zonitoides arboreus	403	5.95	5.13	5.42	4.26	4.67	7.87	8.08	6.25	
Zonitoides nitidus	56	0.81	0.78	0.93	0.62	0.14	0.33	1.01		

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Systematics and evolutionary history of large endemic snails from the Caucasus (*Helix buchii* and *H. goderdziana*) (Helicidae)

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Abstract: Two species of genus *Helix* Linnaeus, 1758 (Mollusca: Gastropoda: Helicidae) endemic to the Caucasus region are known from Georgia and northeastern Turkey: *Helix buchii* Dubois de Montpereux, 1839 and the recently-described but disputed *Helix goderdziana* Mumladze, Tarkhnishvili and Pokryszko, 2008. The latter species is the largest land snail throughout non-tropical Eurasia. We compared shell shapes and genital morphology of the two species. We analyzed mitochondrial COI and nuclear 18S ribosomal RNA and ITS1 gene fragments in 39 specimens of *H. buchii* and *H. goderdziana* from ten locations from the entire distribution range of these species, together with 13 specimens of the widespread *H. lucorum* Linnaeus, 1758 and *H. pomatia* Linnaeus, 1758. Based on shell morphology alone, most of the individuals of the two species can be discriminated using multivariate approaches. The species have different flagellum/diverticulum ratios, and the foot coloration is a fully diagnostic morphological character. Molecular genetic analysis revealed little variation in 18S+ITS1 fragment, and eleven COI haplotypes. Phylogenetic analyses support reciprocal monophyly of *H. buchii* and *H. goderdziana*. The genetic distances significantly correlate with the geographic and morphological distances; correlation of morphological distances with geography is insignificant. The basal lineages of both species are found within two distinct glacial refugia, a result which matches the separation of eastern and western evolutionary lineages of other relicts of the Western Caucasus. The present distribution of *H. goderdziana* coincides with the expected refugial borders, whereas *H. buchii* is likely to have extended its geographical range since the last glaciation.

Key words: Mollusca, phylogeography, DNA, Caucasus, refugia

Helix Linnaeus, 1758 (Gastropoda: Helicidae) are the largest land snails of northern Eurasia. The genus includes over 25 species (Schütt 2005, Welter-Schultes 2009). *Helix buchii* Dubois de Montpereux, 1839, (Figs. 1A, 1C) until recently known as the largest land snail of the western Palaearctic, is an endemic of the mountain broadleaf forests of the Caucasus ecoregion (Zazanashvili *et al.* 2004), which harbor numerous Tertiary relict species and habitats (Tuniyev 1990, Röhrig 1991, Mai 1995, Veith *et al.* 1998, Kikvidze and Ohsawa 1999, Denk *et al.* 2001, Milne and Abbott 2002, Milne 2004, 2006, Zazanashvili *et al.* 2004, Tarkhnishvili *et al.* 2012).

Another large snail, *Helix goderdziana* Mumladze, Tarkhnishvili and Pokryszko, 2008 (Figs. 1B, 1D), has been recently-described from southwestern Georgia near Goderdzi pass (Mumladze *et al.* 2008). This snail is even larger: the shell diameter in some individuals reaches 68 mm (this paper). The distribution ranges of both species overlap, although *H. goderdziana* is limited to the western Lesser Caucasus and is known from only two localities (Fig. 2). Sysoev and Shileyko (2009) disputed the taxonomic status of *H. goderdziana*, suggesting that the traits used in the original description (foot coloration, shell size, and flagellum length) may vary broadly within a species. Indeed, morphological traits in *Helix* are highly variable, and species-level taxonomy is regularly disputed (Schütt 2005, Neubert and Bank 2006, Sysoev and Shileyko 2009).

Delineating species is a common problem in systematics (De Queiroz 2007, Mallet *et al.* 2007, Hausdorf 2010, Mallet 2010), but distinguishing between similar species is a core step to assess and maintain biodiversity (Bickford *et al.* 2006). There is a lack of comprehensive studies on systematics, distribution and conservation of Caucasian *Helix* species. In order to clarify the evolutionary history and taxonomic status of *H. goderdziana* and *H. buchii* (from here onwards – *Endemic Caucasian Helix*, *ECH*), we applied a combination of molecular genetics and morphometric approaches to the samples collected throughout the range of both species. In addition, we provide brief information on the two known localities of *H. goderdziana* to address its conservation status.

MATERIAL AND METHODS

Sampling

During 2008–2010, we collected adult specimens (individuals with well-developed lip) of *Helix buchii* and *H. goderdziana* from Georgia and NE Turkey (Fig. 2). One to twelve *H. buchii* from eight locations, and two to five *H. goderdziana* from both known locations of this species were sampled. The small samples of *H. goderdziana* reflect its rarity. As outgroups for genetic and morphological studies, the widespread species

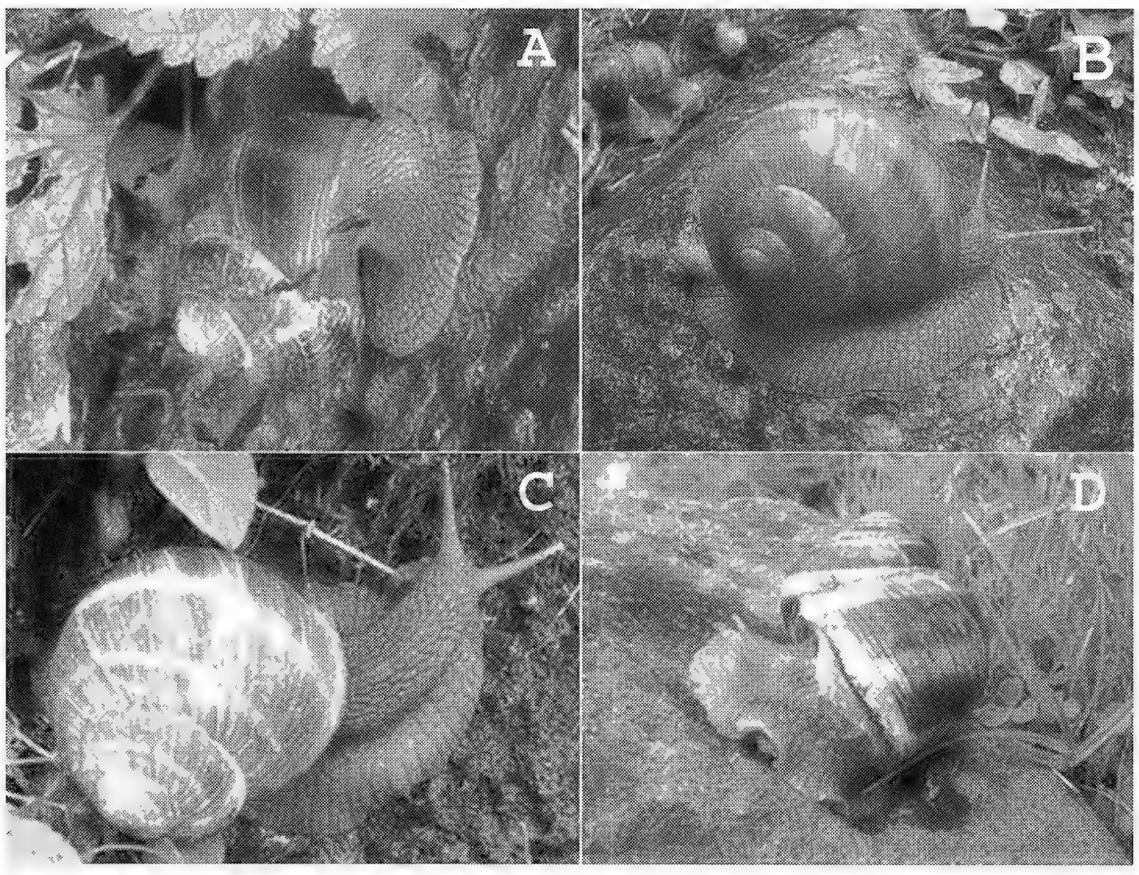

Figure 1. *Endemic Caucasian Helix (ECH).* **A,** subadult *H. buchii*; **B,** subadult *H. goderdziana*; **C,** adult *H. buchii*; **D,** adult *H. goderdziana.*

Helix lucorum Linnaeus, 1758 and *Helix pomatia* Linnaeus, 1758 were used: eight and five adult specimens, respectively (Table 1). Geographic coordinates of each location were recorded with a Garmin Etrex 12 Channel GPS unit (Garmin Corp., Olathe, Kansas, U.S.A.). Live individuals were drowned in water and then preserved and stored in 96% alcohol for further processing. The genitalia were dissected and measured for five *H. buchii*, three *H. goderdziana,* one *H. lucorum,* and one *H. pomatia.* Pieces of muscular tissue of collected individuals were used for DNA extraction and processing. Alcohol-stored specimens and shells are deposited in the collection of Zoological Institute of Ilia State University under accession numbers h1–h59.

Figure 2. Sampled locations of *Helix buchii* (black dots) and *H. goderdziana* (open circles): **1,** Lagodekhi (eastern Greater Caucasus); **2,** Dmanisi; **3,** Didgori; **4,** Borjomi (central Lesser Caucasus); **5,** Khevsha; **6,** Mokhva (central Greater Caucasus); **7,** Bakhmaro (western Lesser Caucasus); **8,** Jamilikhemshin (Kackar Mountains); **9,** Goderdzi Pass (western Lesser Caucasus, type locality of *H. goderdziana*); **10,** Kovanlyk. Outlined area: borders of the Major Forest Refugium (see discussion), *sensu* van Andel and Tzedakis (1996).

Morphology

The shells of adult specimens (thirteen *Helix buchii*, seven *H. goderdziana*, four *H. pomatia*, and four *H. lucorum*) were scanned using a 3D scanner (Roland PICZA 3D Laser Scanner LPX-600). Nineteen landmarks were selected: L0 = intersection of the main axis and the columellar part of lip; L3 = junction of the lip with the body whorl; L6 = apex; other landmarks were positioned using the junctions of two perpendicular planes, the first crossing the landmarks L0, L3, and L6 and the second adjusted perpendicularly to the first so that landmarks L0 and L6 were common to both (Fig. 3). Placing landmarks and extracting coordinates were performed with software Landmark v2.0 (Wiley *et al.* 2005). Geometric morphometry methods are commonly used for the analysis of snail shells (Conde-Padín *et al.* 2007) when landmark data can be captured. However, if the landmarks do not meet true homology criteria, the interpretation of the analysis results might be misleading (Zelditch *et al.* 2004). Because our landmarks (except L3 and L6) cannot be assumed as homologous, we used a "traditional" Principal Component Analysis (PCA; Joliffe 1992, MacCallum *et al.* 1999) for describing shell shape differences using between landmark distances, which are easier to interpret (Blackith and Reyment 1971, Richtsmeier *et al.* 2002).

To maximally approximate the assumptions of PCA and to maintain sufficiently high sample/ variable ratio, we had to reduce the available set of distance measures to few distance variables. Based on visual observations on *Helix buchii* and *H. goderdziana*, most obvious differences in shell shape are due to the shape of shell spire. Consequently, we used the following eight distance measures describing shell spire: L4–L6, L5–L6, L5–L7, L6–L8, L6–L9, L6–L16, L6–L17, L11–L15, L12–L16, and L13–L17 (Fig. 3). In order to meet a normality assumption and minimize size influence and allometric effect, the distances were log-transformed and then standardized residuals of the regression of each character on the distance between shell apex and most proximate distance of outer lip (L1–L6) were calculated, as recommended by Thorpe and Leamy (1983). Standardized residuals calculated for the 10 variables were subjected to Principal Component Analysis (PCA) with components extracted at eigenvalues over 1.

We dissected five adult specimens of *Helix buchii,* three of *H. goderdziana,* one of *H. lucorum*, and one of *H. pomatia* in order to compare qualitative and quantitative traits of their genital morphology. We measured length of flagellum, length of penis + epiphallus, length of bursa tract, diverticulum, maximum length of mucus gland, and length of dart sac of each dissected individual (Fig. 4). All statistical analysis was

Table 1. Sampling locations with GPS coordinates and number of sampled specimens. Abbreviation in brackets for first column stands for: Geo, Georgia; Tu, Turkey; Pol, Poland.

Sampling location	GPS coordinates	Species	DNA samples	Shell samples	Genital samples
Lagodekhi (Geo)	41.85N, 46.29E	*Helix buchii*	1	1	-
Dmanisi (Geo)	41.33N, 44.35E	*H. buchii*	6	2	-
Didgori (Geo)	41.78N, 44.51E	*H. buchii*	7	2	1
Borjomi (Geo)	41.91N, 43.25E	*H. buchii*	2	1	-
Khevsha (Geo)	42.40N, 44.69E	*H. buchii*	1	1	-
Mokhva (Geo)	42.43N, 43.30E	*H. buchii*	12	2	2
Bakhmaro (Geo)	41.89N, 42.37E	*H. buchii*	2	2	-
Jamilihamshin (Tu)	41.14N, 40.93E	*H. buchii*	3	2	2
Goderdzi (Geo)	42.57N, 41.63E	*H. goderdziana*	2	4	2
Kovanlik (Tu)	38.14N, 40.68E	*H. goderdziana*	3	3	1
Tbilisi (Geo)	41.72N, 44.65E	*H. lucorum*	8	4	1
Wroclaw (Pol)	51.11N, 17.01E	*H. pomatia*	5	4	1

performed using SPSS v.16 for Windows (SPSS Inc. Chicago, Illinois, U.S.A.).

DNA analysis and inferring relations between haplotypes

Total cellular DNA was extracted from a small piece of the hind part of the foot of individual snails. Extraction was performed using QIAGEN® QIAamp DNA Mini Kit followed by a slightly modified standard protocol provided by QIAamp DNA Mini Kit Handbook (QIAGEN, Hilden, Germany). Partial sequences of mitochondrial gene COI and fragments of nuclear 18S ribosomal RNA gene and internal transcribed spacer 1b (18S+ITS1) were amplified and sequenced for 34 *Helix buchii,* five *H. goderdziana,* eight *H. lucorum,* and five *H. pomatia.* Amplification conditions and temperature profiles are given in Appendix 1. The amplicons were sequenced on the automatic sequencer ABI 3130 (Applied Biosystems, Foster City, California). Single-stranded sequencing was performed with polymerase chain reaction primers, using the Big-Dye Terminator v3.1 (Applied Biosystems, Foster City, California). DNA sequences were edited using SEQSCAPE v2.5 (Applied Biosystems Inc., Foster City, California); only unique COI and 18S+ITS1 haplotypes were deposited in GenBank (accession # GU784797–GU784807). The alignment of the sequences was performed with BioEdit v7.0 (Hall 1999). Phylogenetic analyses were performed for high-quality sequence fragments including 364 bp for COI (the obtained sequences of COI were not readable in the end of 3′ direction) and 473 bp for 18S+ITS1.

The sequences were aligned with the six most similar GenBank sequences, as shown by BLAST output *Lozekia deubeli* (Kimakowicz, 1890) (COI; GenBank accession # EU182503), *Marmorana scabriuscula* (Deshayes, 1830) (COI; # EU189930), *Arianta arbustorum* Linnaeus, 1758 (both genes; # AF296946 and AY546455), species of *Satsuma* H. Adams, 1868 (both genes; #AB242535 and AB481049), and *Iberus* Montfort, 1810 spp. (both genes; # EF440266 and EU446026), and *Caucasotachea calligera* (Dubois de Montpereux, 1840) (18S+ITS1; # GU784810 – sequenced by authors specifically for this manuscript). Unfortunately, no homologous DNA fragments of other *Helix* are available from GenBank). Phylogenetic relationships between the individual COI haplotypes were inferred

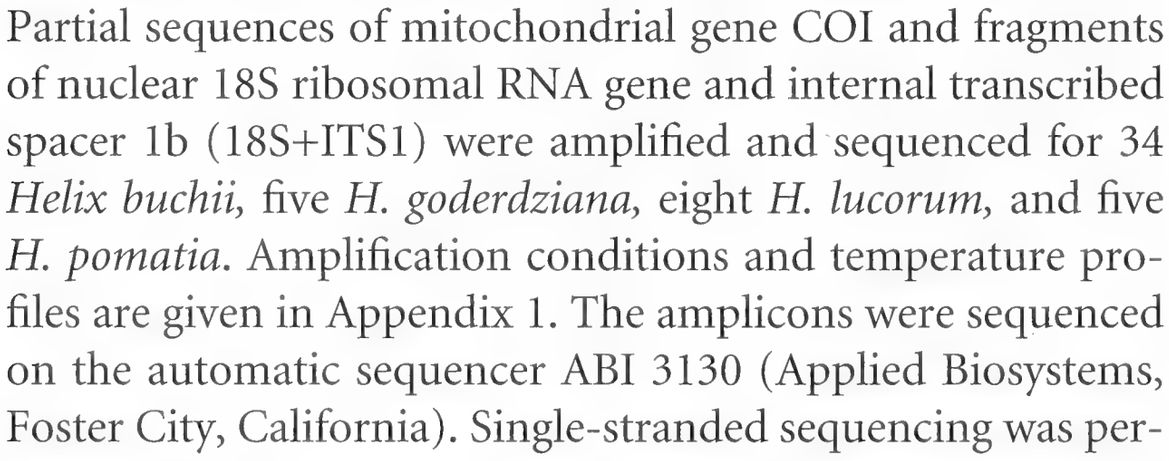

Figure 3. The position of the landmarks used for morphometric analysis of shells of the studied species.

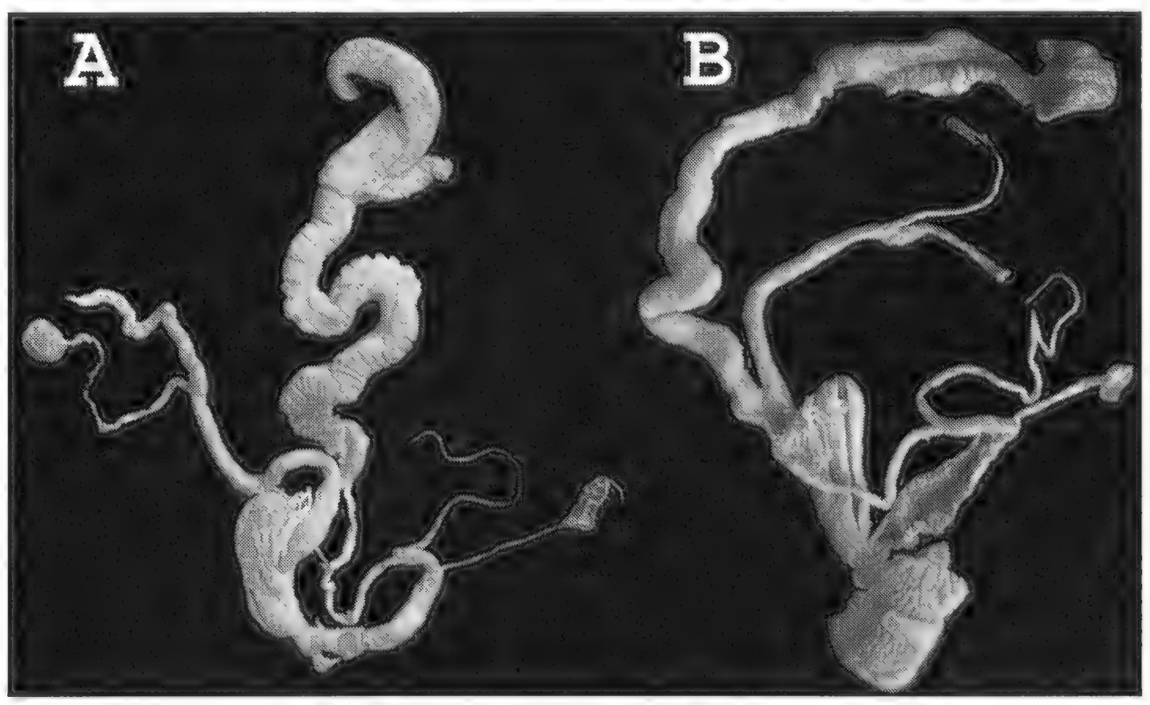

Figure 4. Overall view of genital organs. **A,** *Helix goderdziana*; **B,** *H. buchii.*

with neighbor-joining (NJ), maximum parsimony (MP), and Bayesian algorithms. NJ and MP trees were inferred using MEGA v5 (Tamura *et al.* 2011) with applying default settings (all positions included, 1000 bootstrap replications, Max-mini branch-and-bound for MP). Bayesian phylogenetic analysis was performed using the software BEAST v1.5.1 (Drummond and Rambaut 2007). Posterior distributions of parameters were approximated using Markov Chain Monte Carlo (MCMC) with length of chain 3×10^7 that harvested effective sample size (ESS) > 100 for each parameter. The best model was identified by the model comparison procedure based on the marginal likelihood, using a code written for BEAST (Suchard *et al.* 2001). Prior to this analysis, we tested the molecular clock hypothesis (Hasegawa *et al.* 1985) and found the best model of nucleotide substitution using Bayesian Information Criterion (BIC) using software MEGA v5 (Tamura *et al.* 2011). All possible evolutionary pathways among the obtained haplotypes of *H. buchii* and *H. goderdziana* were reconstructed using Median-Joining (MJ) algorithm (Donnelly and Tavare 1986, Bandelt *et al.* 1999) using the software Network 4.6.1 (Bandelt *et al.* 1999). The GenGIS software (Parks *et al.* 2009) was used for plotting the phylogenetic tree on a geographic map (Fig.7).

Because 18S+ITS1 sequences were identical for three out of four studied species (see results), they were not subjected to the detailed phylogenetic analyses.

To explore to what extent morphological variability among *ECH* individuals is associated with their evolutionary differentiation we applied partial Mantel test (Manel *et al.* 2003) with 10,000 permutations, using IBD software (Bohonak 2002). All 20 studied *ECH* individuals were included in the analysis, without *a priori* attribution to *Helix buchii* or *H. goderdziana*. To perform Mantel test genetic distances between individual COI sequences were estimated according to Kimura (1980) using MEGA v5.

Morphological distances (shell shape) were estimated as Euclidean distances based on individual scores from all PCA axes with eigenvalues exceeding unity. We explored whether: (I) genetic distances between the individuals of *Helix buchii* and *H. goderdziana* significantly correlated with geographic distances between the locations; (II) morphological distances significantly correlated with (a) genetic distances between the individuals, and (b) geographic distances between the locations.

RESULTS

Morphometry

The output of PCA based on the shell measurements is shown in Table 2 and Fig. 5. Two PCA axes were extracted with eigenvalues > 1. All included variables had a high communality values (> 0.8), indicating that the result can be used in a meaningful way (Table 2). The first PCA axis (72% of the total variation and 10% for second PCA axis) had similar positive loading for all the variables which implies that increasing score values along the axis marks higher shells with broader spire (wider apical whorls) relative to the shell size. Adult individuals of *Helix lucorum* have the highest scores along this axis, and *H. buchii* and *H. pomatia* have the lowest scores. *Helix goderdziana* keeps an intermediate position between *H. buchii* and *H. lucorum*, but the overlap is higher with the latter species (Fig. 5). The interspecific differences in the average values of the first PCA scores are significant

Table 2. Loadings of individual shell dimensions on the PCA axes. PCs with eigenvalues exceeding unity are shown. All variables are standardized residuals of the corresponding measurements from the regression line on lnL1–L6. Last column contains Communality (indicating a percent of variance accounted by the PCs) values for each distance variable.

Distances	PC1	PC2	Communalities
L4–L6	0.91	-0.27	0.894
L5–L7	0.88	-0.20	0.811
L5–L6	0.82	-0.34	0.779
L12–L16	0.83	0.50	0.932
L6–L8	0.90	0.09	0.813
L6–L9	0.91	-0.05	0.828
L6–L15	0.65	-0.21	0.465
L6–L16	0.88	-0.26	0.837
L6–L17	0.90	-0.18	0.838
L11–L15	0.88	0.34	0.897
L13–L17	0.75	0.63	0.956

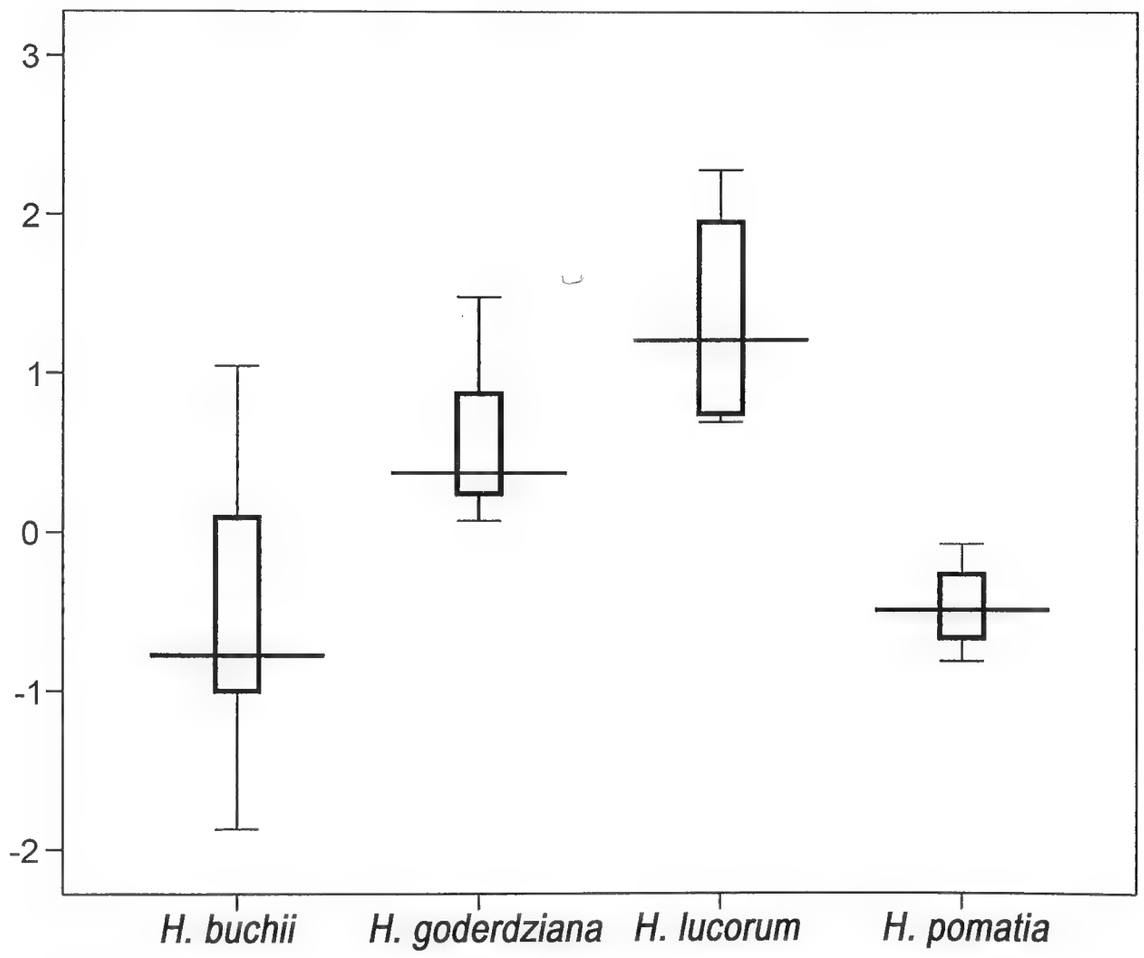

Figure 5. Box plots of individual scores of the four studied *Helix* species along the first PCA axis defined by shape of the shell spire.

Table 3. Multiple pairwise comparison (with Bonferroni adjustment) after One-way ANOVA based on individual scores for first PCA axis. Numbers indicate the mean differences. Numbers in bold represent significant results at 0.05 significance level.

	H. goderdziana	*H. lucorum*	*H. pomatia*
Helix buchii	**-1.14**	**-1.87**	0.42
H. goderdziana		-0.76	1.07
H. lucorum			**1.82**

(One-way ANOVA, $F_{3,26} = 8.9$, $P < 0.001$). Mean differences are significant ($P < 0.05$ after Bonferroni adjustment) between *H. lucorum* and *H. buchii*, *H. lucorum* and *H. pomatia*, *H. buchii* and *H. goderdziana*; the differences are not significant ($P > 0.05$) between *H. goderdziana* and *H. lucorum*, *H. pomatia* with *H. buchii*, and *H. pomatia* with *H. goderdziana* (Table 3).

Most of the genitalia measurements did not show obvious differences neither between *Helix buchii* and *H. goderdziana*, nor among *ECH* and the two other *Helix* species (Fig. 6). However, the flagellum/diverticulum ratio in the studied individuals of *H. goderdziana* was significantly lower than in *H. buchii* and much shorter in *ECH* than in either *H. lucorum* or *H. pomatia*.

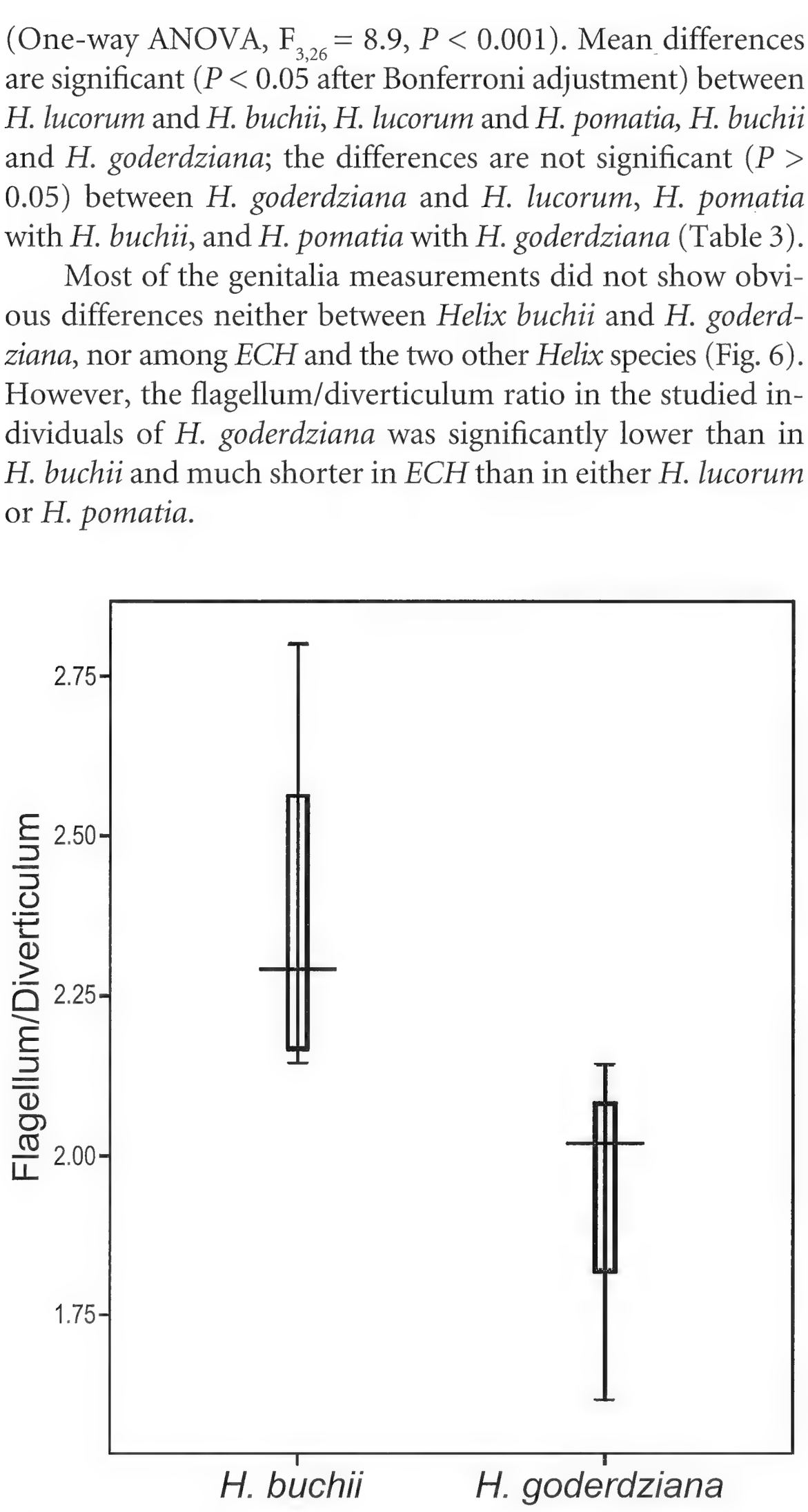

Figure 6. Box plots of flagellum/diverticulum ratios for *Helix buchii and H. goderdziana*.

Phylogenetic relations of the studied species

The sequenced fragment of nuclear 18S+ITS1 was identical for *Helix goderdziana, H. buchii* and *H. lucorum*. Five substitutions separate these species from *H. pomatia*. The sequenced COI fragment had 92 informative sites for all 52 obtained sequences of four *Helix* species. The lowest BIC value was shown for Hasegawa-Kishino-Yano model (HKY) with gamma distribution (HKY+G). Five haplotypes of *H. buchii*, two of *H. goderdziana*, three of *H. lucorum* and one of *H. pomatia* were identified. Individual haplotypes of *ECH* differed by 1–15 substitutions. NJ, Bayesian, and MP consensus tree (Fig. 7) supported (1) monophyletic origin of *ECH*, with respect to the outgroup taxa (*H. pomatia, H. lucorum,* one hygromiid and four helicids downloaded from the GenBank) and (2) reciprocal monophyly of *H. buchii* and *H. goderdziana*. The MJ network (Fig. 8) showed a single possible path connecting *H. goderdziana* and *H. buchii*. Six out of the seven unique haplotypes inferred within *ECH* are geographically distinct. Two haplotypes of *H. goderdziana* are attributed to NE Turkey (Kovanlyk) and SW Georgia (Goderdzi), respectively; two haplotypes of *H. buchii* are attributed to the Central Greater Caucasus (Mokva, Khevsha) and to the Lesser and Eastern Greater Caucasus (Borjomi, Didgori, Dmanisi, Lagodekhi) respectively. Three remaining basal haplotypes of *H. buchii* mark individual locations in the Western Lesser Caucasus (Jamili, Bakhmaro). Only the latter location had two closely-related haplotypes, individuals from other studied ECH locations did not differ genetically. The hypothesis of a molecular clock was supported (LRT = 56.8, $P < 0.001$) for the sequenced fragment of COI, without considering the codon position.

Relationships between morphology, genetics, and geography

A Mantel test showed significant correlation between genetic and geographic distances for *ECH* samples ($r_{xy} = 0.41$, $P < 0.001$). The morphological distance (distance between the individuals based on the first two PCA axes for shell measurements) significantly correlates with genetic distance (COI sequence) between the corresponding individuals, if controlled for geographic distance ($r_{xy} = 0.22$, $P = 0.02$) between the locations but no correlation of morphological distance with geography was detected.

DISCUSSION

Systematics and Taxonomic inference

This study suggests that *Helix buchii* and *H. goderdziana* are two distinct, reciprocally-monophyletic evolutionary lineages. Morphological differences between these species are slight but obvious. Foot coloration, albeit variable in most

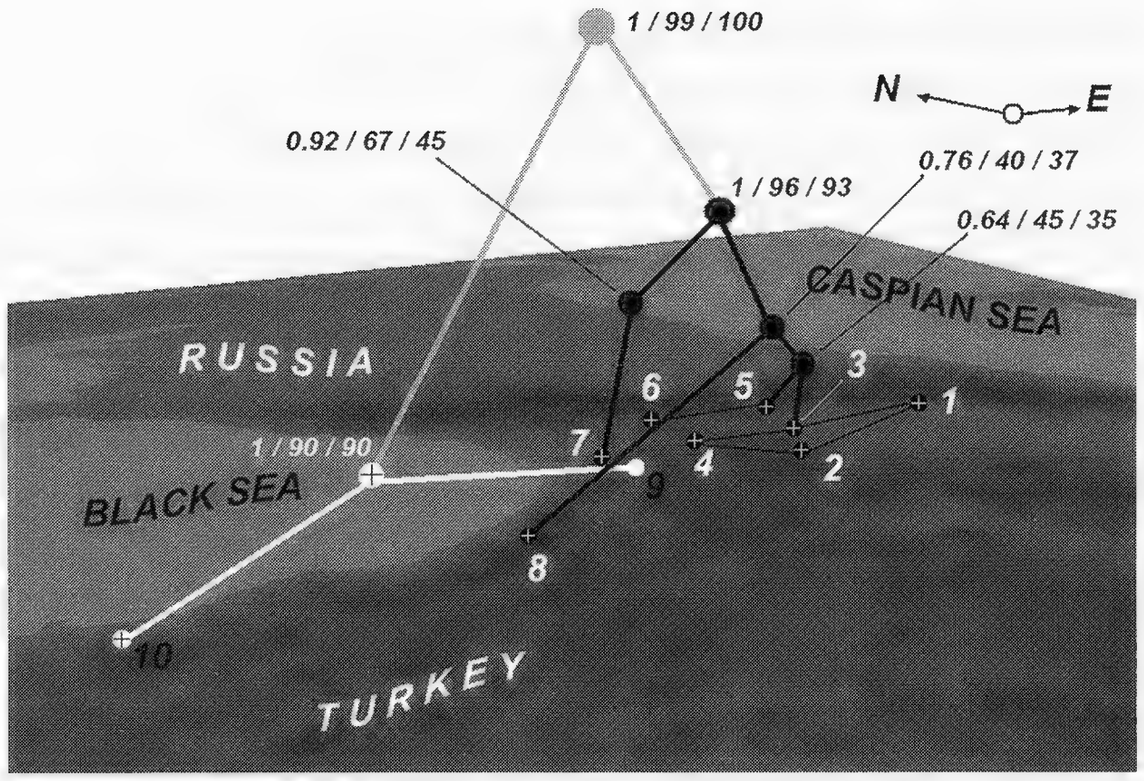

Figure 7. Phylogenetic relations between the *ECH* from different parts of the range; consensus tree based on the BA, NJ, and MP analyses. *Helix buchii*: black lines and circles; *H. goderdziana*: white lines and circles. The numbers attributed to individual nodes are Bayesian posterior probabilities / bootstrap supports for NJ tree nodes / bootstrap supports for MP tree nodes. Numbering of the sites (smaller crossed circles on the map) as in Fig. 2. The sites with identical haplotypes of *H. buchii* (1–4 and 5–6) are connected with narrower lines. Note that site 7 unites two haplotypes (see results).

land snails (Sysoev and Schileyko 2009), is the fully diagnostic character. In over 100 observed live individuals of *H. buchii*, the foot is dark, from grey to black, whereas over 20 adult and juvenile *H. goderdziana* found in both natural locations had light-colored yellowish foot, similar to that of the widespread *H. lucorum* (not all the observed specimens were used in the analysis, see Table 1). *Helix goderdziana* have in average larger shells with relatively broader spires than *H. buchii*, being more similar in shell shape to *H. lucorum* than to its sister species, if size and allometry factors are assumed. At last, *H. goderdziana* have lower flagellum/diverticulum ratio than *H. buchii*, and both *ECH* species have substantially lower diverticulum/flagellum ratio than *H. lucorum* or *H. pomatia*.

Long-running debates on the species criteria focus on some questions, on which an expert consensus perhaps never will be achieved (*e.g.*, Mayden 1977, Hey 2001, Avise 2004, de Queiroz 2007, Hausdorf 2010, Mallet 2010). Incipient species commonly exchange genes for millions of years, although this might not prevent progressive divergence (Mallet *et al.* 2007, Hausdorf 2010). We follow the suggestion of Mallet (2010) and refrain from the puritanical approach to species definition, deciding the nomenclatural questions dependent on the practical appropriateness. *Helix goderdziana* and *H. buchii* are morphologically, ecologically and geographically distinct and they are marked with reciprocally-monophyletic mitochondrial haplo-groups. These facts convince the authors that the differential species names are practically applicable to the studied taxa.

Evolutionary history of Endemic Caucasian Snails

If we consider morphological similarity, geographic closeness, and monophyly (based on COI sequence) of *ECH* relative to the analyzed widespread *Helix* species, *H. buchii* and *H. goderdziana* are likely to be sister taxa, although this assumption needs additional genetic data for more representatives of the genus.

Helix lucorum (and not the superficially more similar *H. pomatia*) is genetically closer to the *ECH* clade. This is supported by both phylogenetic inference based on the mitochondrial COI and structural identity of the sequenced fragment of nuclear 18S+ITS1. As opposed to the suggestion of Steinke *et al.* (2004), the fragment is less variable among the included outgroup of Helicidae than the sequenced fragment of COI: the mean proportion of pairwise differences among *H. buchii*, *H. lucorum*, and species of the outgoup reach 0.12 for the homologous 18S+ITS1 fragment but 0.23 for homologous fragment of mitochondrial COI.

The outcome of the partial Mantel test suggests that size and shape of shell correlates with genetic distance for *ECH* rather than by short-term/reversible

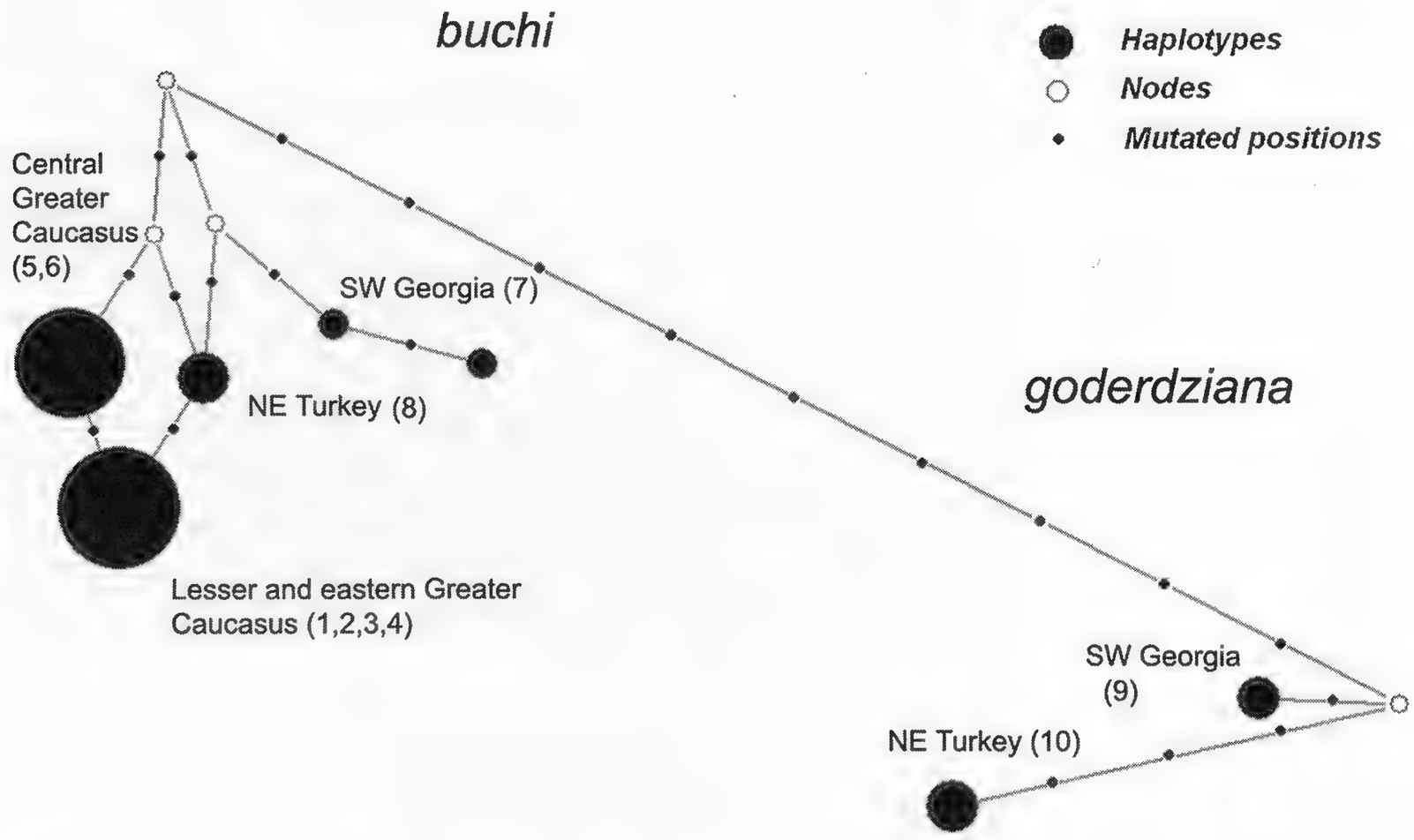

Figure 8. Median-joining network connecting inferred haplotypes of *Helix goderdziana* (Goderdzi and Kovanlyk) and *H. buchii* (all others). Numbers in parenthesis represent location numbers (see Fig. 2). Size of the circles marking haplotypes is proportional to the number of respective individuals.

adaptations to local climates. The extant range of *Helix goderdziana* is restricted to the western Lesser Caucasus in SW Georgia and NE Turkey. Paleontological data suggests that this area supported a major forest refugium (MFR) during the last glacial maxima (Zeist and Bottema 1988, Van Andel and Tzedakis 1996). Molecular genetic study of the salamander *Mertensiella caucasica* (Waga, 1876) (Tarkhnishvili *et al.* 2000) revealed presence of two distinct evolutionary lineages of the salamanders isolated since pre-glacial time. The range of the western lineage coincides with the MFR and, hence, with the distribution range of *H. goderdziana*; the range of the eastern lineage is restricted to a small area in central Georgia. This finding supports the hypothesis of existence of multiple forested refugia east of MFR (Velichko and Kurenkova 1990, Tarkhnishvili *et al.* 2012). The geographic line separating MFR from the habitats supporting the eastern lineage of the salamander and the basal haplotype of *H. buchii* coincides with a belt of dry climate crossing the Meskheti Mountains in SW Georgia (Tarkhnishvili *et al.* 2008). The present geographic distribution of the climates was shaped ca. 6 MYBP (million years before present) (Fortelius *et al.* 2002, but see Micheels *et al.* 2009). Data on the rates of molecular evolution in COI in mollusks are controversial. Marko (2002) suggests 1.21% substitution rates per MY, but later studies of snail divergence in Europe (Gittenberger *et al.* 2004, Haase and Misof 2008) indicate that the molecular evolution can be much faster. If Marko's calibration is considered, the average split time between *H. buchii* and *H. goderdziana* may be 3.36 MYBP (95% confidence interval 1.7–4.5 MYBP). However, one cannot exclude that the lineages have been separated much later, in middle or even late Pleistocene. One can suppose that the "dry belt", limiting the eastern range of *H. goderdziana*, was an insuperable barrier for the spread of mesophylic species with limited dispersal ability during glacial maxima. This may have triggered the original split between the two snail lineages. The ancestral lineage of *H. buchii* survived in the refugia far from the Black Sea with a more continental climate, and the ancestors of *H. goderdziana* survived in MFR.

Habitat preferences and conservation

There are remarkable ecological differences between the two *ECH* species. *Helix buchii* is found in a wide habitat spectrum, mainly broadleaf forest litter away from the water sources but never in coniferous forest. This species is relatively common in primary forests of Caucasian mountain, whereas both known locations of *H. goderdziana* lay in exceedingly damp habitats along the brooks in mixed or broadleaf forest (*Alnus barbata* and *Picea orientalis*). The only known Georgian locality of *H. goderdziana* is currently under intensive anthropogenic pressure. In the last 5 years, the habitat was repeatedly littered and damaged (most of trees were cut down), and water in the brooks was polluted by sawdust and waste. We were unable to find *H. goderdziana* in 2010 and 2011 at the type locality. The disappearance of the species may be related either to the changing of microclimatic conditions at the brooks or the water pollution. The potential solution for the future is creation of a mini-reserve in the area, but this needs immediate attention from the relevant governmental bodies and international conservation community.

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Appendix 1.

Source	Primer sequence	Amplification conditions	Temperature profile
COI universal (Folmer *et al.* 1994)	5′-GGTCAACAATCATAAAGATATTGG-3′ 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	20µl total volume, with: 2 µl template DNA 1.5U of Taq polymerase (Promega) 1x Promega buffer 1.5 µm of $MgCl_2$ 0.1 µm of each dNTP, 0.1 µm primer concentrations	1 cycle of 3 min @ 95 °C 25 cycles: 40s @ 94 °C 40s @ 50 °C 1min @ 72 °C 1 cycle of 10 min @ 72 °C
18S+ITS1 mollusc-specific (Armbruster *et al.* 2000; van Moorsel *et al.* 2000)	5′-TAACAAGGTTTCCGTAGGTGAA-3′ 5′-GCTGCGTTCTTCATCGATGC-3′	20 µl total volume, with: 3 µl template DNA 1U of Taq polymerase(Promega) 1x romegabuffer 1.5 µm of $MgCl_2$ 0.1 µm of each dNTP, 0.1 µm primer concentrations	1 cycle of 3 min @ 95 °C 25 cycles: 40s @ 94 °C 30s @ 56 °C 0.3 °C each cycle) 1min @ 72 °C 1 cycle of 10 min @ 72 °C

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Mating in *Veronicella sloanii* (Cuvier, 1817) (Veronicellidae)

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Abstract: The systellommatophoran slug *Veronicella sloanii* (Cuvier, 1817), is a simultaneous hermaphrodite. This slug is an agricultural and horticultural pest in Barbados and several islands of the Lesser Antilles. Over the period January–July 2006 and June–August 2010, the mating behavior of this species was determined by *ad libitum* and focal animal sampling of captive slugs collected from six sites on the island of Barbados, supplemented by observations and length measurements of slugs seen mating in the field. Individuals of *Veronicella sloanii* mated reciprocally in pairs, but also in a multi-partner ring formation involving three individuals. Two stages in the mating process were identified, courtship and copulation. Courtship was short, less than two minutes in mating events that led directly to copulation (mean 1.87 minutes, range 0.25–2, $N = 53$). Copulation in contrast was long, lasting on average 1.03 hours (range 0.4–2, $N = 40$). During mating the penial gland of each partner made contact with the foot or the hyponotum of the other partner. Aggressive behavior during mating in this slug was manifested by non-mating individuals pushing themselves between mating pairs resulting in the withdrawal of the penis of the mating pairs and cessation of copulation. A strong size-assortative mating pattern was observed; individuals in mating pairs were of similar size.

Key words: mating ring, reciprocal copulation, simultaneous hermaphrodite, Systellommatophora, terrestrial slug

Veronicella sloanii (Cuvier, 1817) was first reported from Jamaica (Thomé 1988), but has since spread to Barbados and several islands in the Lesser Antilles archipelago, where it is known as a pest of agricultural and horticultural crops (Fields and Robinson 2004). In Barbados, the species may be found in gardens, plant nurseries, and farms as well as in more natural environments such as woods and gullies. *Veronicella sloanii* belongs to the Clade Systellommatophora, Family Veronicellidae (Bouchet and Rocroi 2005). In the literature both *Veronicella sloanii* and *Veronicella sloanei* have been used to refer to this species, however the original spelling "onchid. Sloanii" (Cuvier, 1817) has precedence and is used here. The reproductive system of veronicellid slugs exhibits the diaulic condition, where distal to the carrefour, the 'male' and 'female' ducts are separate (Gómez 2001). Thus, in *V. sloanii* there are two genital pores; the male genital pore located at the base of the right tentacle and the female genital pore located roughly midway along the right hyponotum. Associated with the penis, is a penial gland also termed the digitiform gland (Peterellis and Dundee 1969) or the dart gland (Rueda 1989) the function of which is unknown. *Veronicella sloanii* is a simultaneous hermaphrodite.

Mating behaviors in sexual reproducing gonochorists are underlain by selective forces acting differently on males and females (Bateman 1948) and this was later shown to apply to hermaphrodites as well (Charnov 1979). Thus within mating systems one would expect to see behaviors that maximize an individual's reproductive fitness (Alcock 1994, Michiels 1998) such as a preference for large more fecund females (Ridley 1983, Crespi 1989, Charnov 1996, DeWitt 1996, Yusa 1996, Harari *et al.* 1999, Koene *et al.* 2007) and male-male competition (Crespi 1989, Michiels 1998, Wong and Condolin 2005). Mate choice based on size can lead to homogamy or size assortative mating (Ridley 1983, McLain 1984, Crespi 1989, Rowe and Arnqvist 1996, Koene *et al.* 2007, Chase *et al.* 2010). Size-assortative mating has been reported in a wide range of taxa including planarians, earthworms, arthropods and fish (Anthes 2010). In molluscs, a positive relationship between body sizes of copulating individuals has been reported in marine (Crozier 1917, 1918, Angeloni and Bradbury 1999, Angeloni *et al.* 2003, Yusa 1996) and freshwater (Staub and Ribi 1995, Chase *et al.* 2010) species. In terrestrial molluscs a trend towards size-assortative mating was found in some species but not in others (Baur 1992, Tomiyama 1996, Jordaens *et al.* 2005). Male-male competition may occur prior to copulation, during copulation or after copulation (Parker 1970, Alcock 1994) and can be manifested in behaviors such as male to male conflict and mate guarding (Eberhard 1998).

Much has been reported on the mating behaviors of stylommatophoran slugs such as the agriolimacids (Karlin and Bacon 1961, Reise 1995, Reise *et al.* 2007, Hutchinson and Reise 2009), the ariolimacids (Heath 1916, Leonard *et al.* 2002), the arionoids (Kozlowski and Sioner 2001, Kozlowski 2007, Dreijers *et al.* 2013), the limacids (Karlin and Bacon 1961, Langlois 1965) and the milacids (Karlin and Bacon

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1961, Forcardi and Quattrini 1972, Barker 1999). In comparison, there is a paucity of information on mating behaviors in veronicellid slugs. Descriptions of mating have been reported for *Veronicella tenax* Baker, 1931 by Rivero (1946), *Diplosolenodes* (Thomé, 1976) by Gerhardt (1937), *Phyllocaulis soleiformis* (d'Orbigny, 1935) by Thomé 1968, Thomé *et al.* 1999), *Phyllocaulis boraceiensis* (Thomé, 1976) by Thomé *et al.* (1999), *Sarasinula plebeia* (Fischer, 1868), *Belocaulis angustipes* (Heynemann, 1885) and *Leidyula floridana* (Leidy, 1851) by Rueda (1989) and *Sarasinula linguaeformis* (Semper, 1885) and *S. plebeia* by Mansur and Thomé (1994). Some of the descriptions are based on serendipitous observations of a single mating event (Gerhardt 1937, Thomé *et al.* 1999), or of the mating behavior of five slugs on one night (Rueda 1989). For other species the sample size or duration of study is not given. Thus, though this information is valuable it may not represent the full extent of the mating behaviors of the species described.

By describing the details of the mating behaviors of *Veronicella sloanii* we aim to contribute to an understanding of the biology of veronicellids, a highly invasive pest group.

MATERIALS AND METHODS

Laboratory studies

The study took place over a nine month period from January 2006–July 2006 and June–August 2010 on slugs held captive in the laboratory. During these periods, slugs were collected from a variety of habitats in Barbados, including residential gardens at Mangrove Park (N13°05′58″,W59°28′15″) and Long Bay (13°07′48.2″ N, 59°26′23.0″ W), wooded areas at Checker Hall (13°16′47″ N, 59°38′44″ W), and in The Belle Gully (13°06′40.7″ N, 59°35′22.3″ W), Fairview (13°09′05.0″ N, 59°32′32.9″ W), and Coffee Gully (13°10′45.3″ N, 59°32′59.3″ W). The animals were held in groups of ten within aquaria (315 x 145 x 150 mm) the bottom of which contained loosely packed soil to a depth of about 65 mm. The aquaria were kept in a laboratory at The University of the West Indies and in a garden shed in a residential garden under natural lighting conditions. Slugs were monitored for two weeks before being replaced with newly collected individuals. Lettuce, *Hibiscus* leaves, Irish potato, carrots and cucumber were provided as a food source. Housing units were cleaned twice a week.

Ad libitum and focal animal sampling of the slugs were conducted over a cumulative period of 98 hours to determine the times when the slugs were most active, the types of behaviors exhibited during mating and the sequence of these behaviors. Digital video recordings made using a JVC Hard disk camcorder GZ-MG30U on all study nights were used to supplement direct observations. Slugs were found to be most active between 6:30 p.m. and 5:00 a.m., and focal animal studies were subsequently conducted during this period to determine the durations of behaviors seen in mating. Duration of courtship events were graphed on a frequency histogram.

Field observations

Information on mating gathered from captive slugs was supplemented by observations made when slugs *in copula* were encountered in the field. These mating individuals were taken to the laboratory where their lengths were recorded by placing them on a glass sheet and allowing them to crawl freely. Measurements were taken when an individual was fully extended. The data were used to compare the sizes of mating partners.

RESULTS

Eighty-eight instances of mating in *Veronicella sloanii* were observed during the period of study. Mating individuals were observed on the soil and as well as on the sides and glass cover of the aquarium. Mating was simultaneously reciprocal in 77% of these events with each individual in a mating pair adopting a "U" shape with its head tucked beneath the other slug, midway along the body. In the other cases, copulation involved three individuals (A, B, and C) arranged in a closed chain where the penis of A intromits into the female genital pore of B, that of B intromits into the female genital pore of C and that of C intromits into the female genital pore of A.

The description of couplet mating is based on 40 cases of pair mating observed from onset to termination and 53 courtship events. Two stages of the mating process in *Veronicella sloanii* were identified, courtship and copulation. Courtship involved three stages here identified as the approach, the initial contact and hyponotum lifting. In the approach phase, individuals moved towards each other head-to-head (70%, $N = 53$), or one individual approached another from the side (30%, $N = 53$) (Fig. 1A). Initial contact was made in one of three ways. A slug used its ocular and tactile tentacles to rub the head and anterior-most region of the notum of the other individual (64%, $N = 53$), or brushed the right side of its body along the right side of another slug (28%, $N = 53$) or pushed its head beneath the body of another slug in the region of the female genital pore (8%, $N = 53$) (Fig. 1B). During tentacle rubbing, an individual would often raise the anterior-most region of its body to access the notum of the other slug. Tentacle rubbing was predominantly reciprocal (74 %). Brushing of the body arose when slugs approached head-to-head but, just before contact veered slightly to the side so that the sides of their bodies made first contact rather than their tentacles. The final stage of courtship, hyponotum lifting, occurred soon after initial contact. The area around the female genital pore became swollen and the hyponotum around this area was lifted from the travel surface. As initial contact was made,

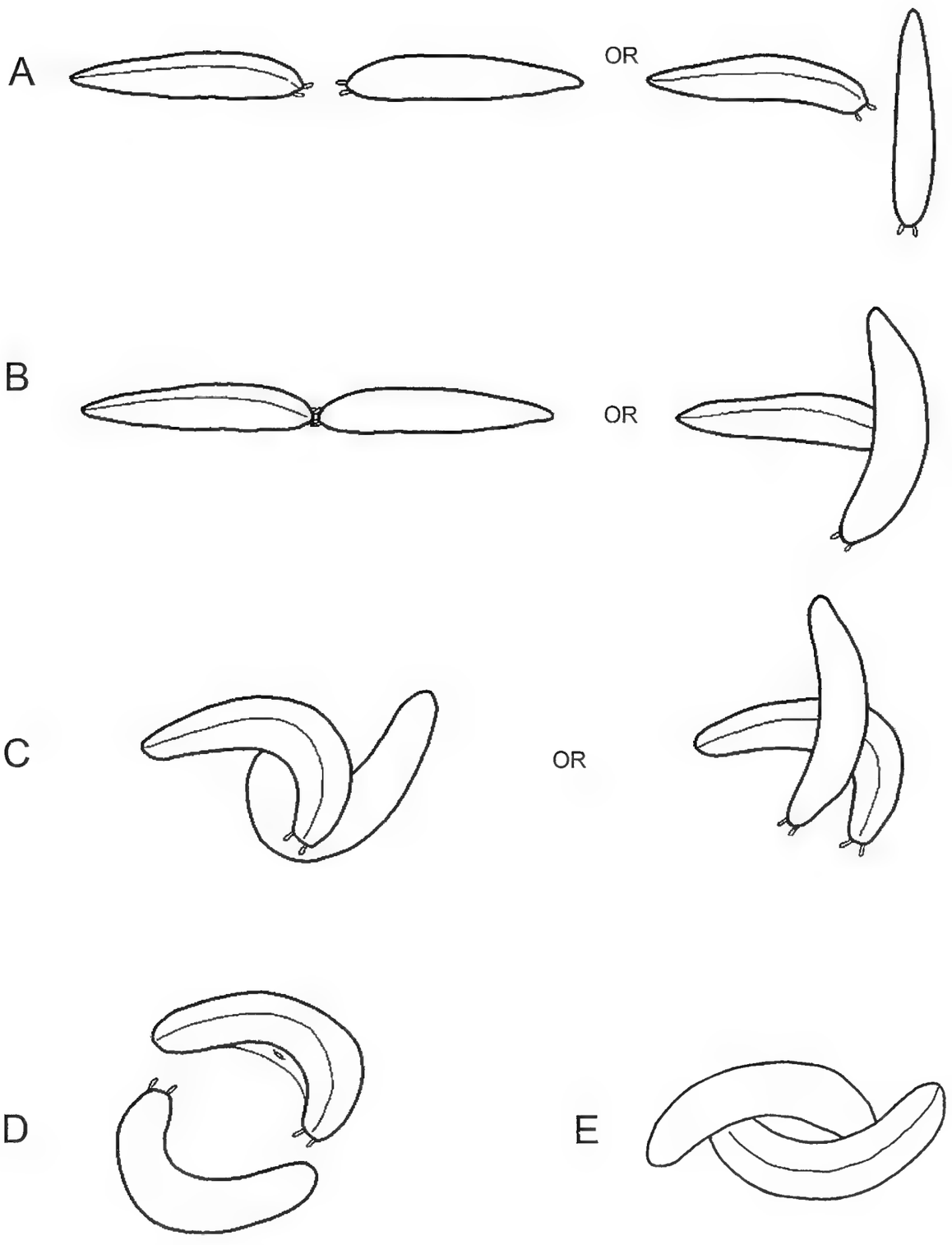

Figure 1. Stages of couplet formation in *Veronicella sloanii.* **A,** Slugs approach each other; **B,** Initial contact; **C,** Body brushing; **D,** Slugs with raised hyponota; **E,** Slugs *in copula.*

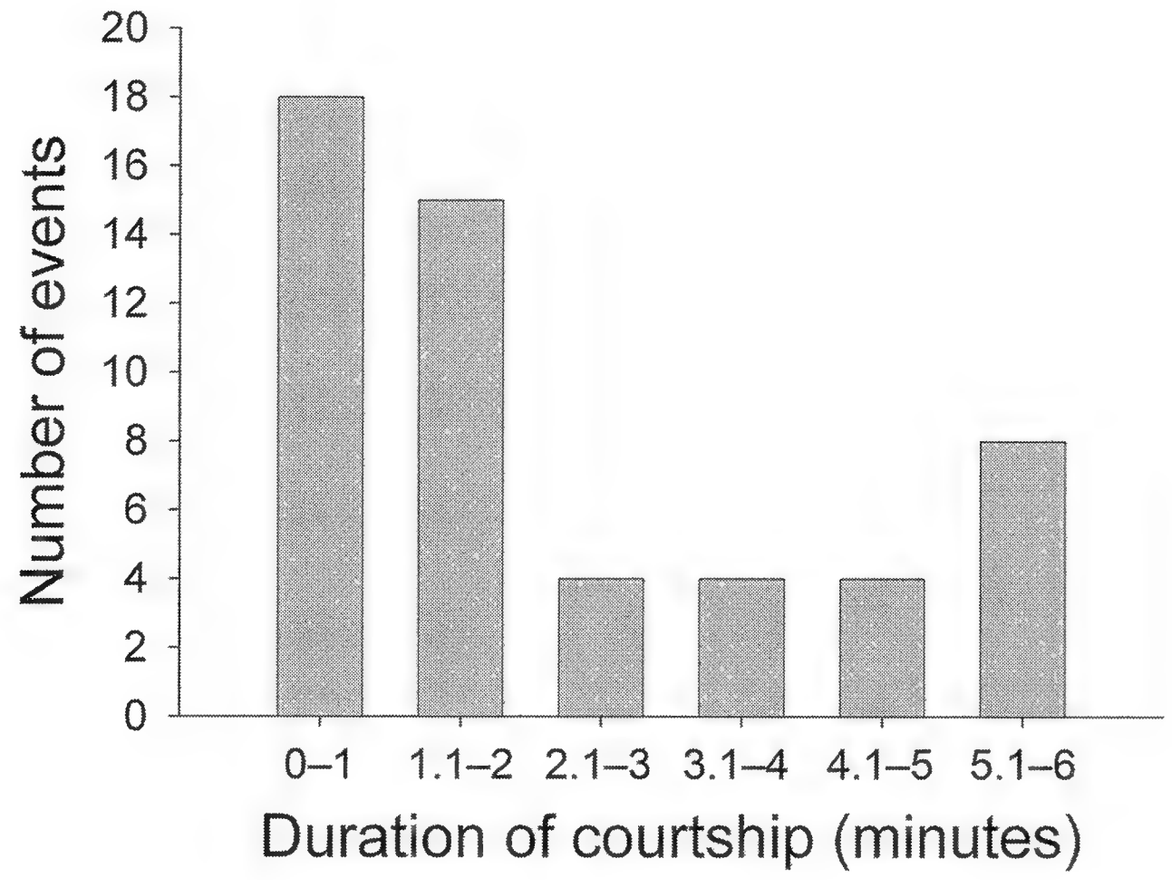

Figure 2. Duration of courtship in *Veronicella sloanii.*

the slugs commenced circling, *i.e.*, slowly traversing a circle in place while brushing the right sides of their bodies against each other (Fig. 1C). When initial contact was made in the region of the genital pore, the circle traversed was larger (Fig. 1D) but, as the slugs continued to circle, subsequent turns became tighter, decreasing the space between the two slugs, and when they touched, brushing of the bodies began. The individuals then dipped their heads beneath their partner's hyponotum in the region of the genital pore and ceased circling. On occasions where the slugs were positioned on the sides of the aquarium, intromission of the penis was seen at this point in the mating process. Thus, this tucked position and lack of motion was taken as a sign that courtship had ended and that copulation had commenced. Of 53 courtship events observed, 25% did not terminate in copulation.

The duration of courtship ranged between 0.25 and 6 minutes. A frequency plot of the time spent in courtship was bimodal (Fig. 2). Courtship events that lasted less than two minutes were the ones that terminated in copulation (62%) and on average lasted 0.8 minutes (*SD* 0.43 minutes). In 80% of cases where courtship exceeded two minutes, the slugs were observed moving rapidly around the aquarium, exhibiting a raised hyponotum. Some of these individuals, on encountering other slugs, would use their tactile tentacles to rub the mantle of these slugs and 50% of these encounters terminated *in copula.*

During copulation, the two slugs lie next to each other, facing opposite directions, with their bodies curved and the head of each tucked under its partner midway along the hyponotum (Fig.1E.). As copulation proceeded the head might reappear from beneath the hyponotum of its mate (Fig. 3). The mean duration of copulation, in the 40 cases that were observed from start to finish, was 1.03 hours (range 0.4–2).

Pairs of slugs *in copula* were found in close proximity to each other, often touching, giving rise to aggregates of slugs in a localized area of the aquarium ($N = 7$). Included in these aggregates were non-mating individuals that lay motionless next to the mating pairs or crawled between mating slugs. On four occasions, one of these wandering individuals copulated with an individual that had recently terminated copulation. In these instances only the circling and brushing phases of courtship were seen. These non-mating individuals would also occasionally interrupt a mating pair ($N = 10$) by pushing against one or both of the slugs, stretching the penis of one or both of the mating individuals. The penis was seen lying over the notum of the interloper (80%) or was dislodged from the female genital pore of its mate (20%).

The penial gland of a slug emerged periodically during copulation and made contact with the foot or hyponotum of its mating partner either in a stabbing or stroking motion (Fig. 4). On one occasion, when a slug inserted itself between

Figure 3. Mating couplet.

a mating couplet the penial papilla of one of the pair made contact with the body of the interloper rather than that of its partner. Stroking of the interloper instead of the partner resulted, and after six minutes the mate that was not being stroked began to withdraw its penis.

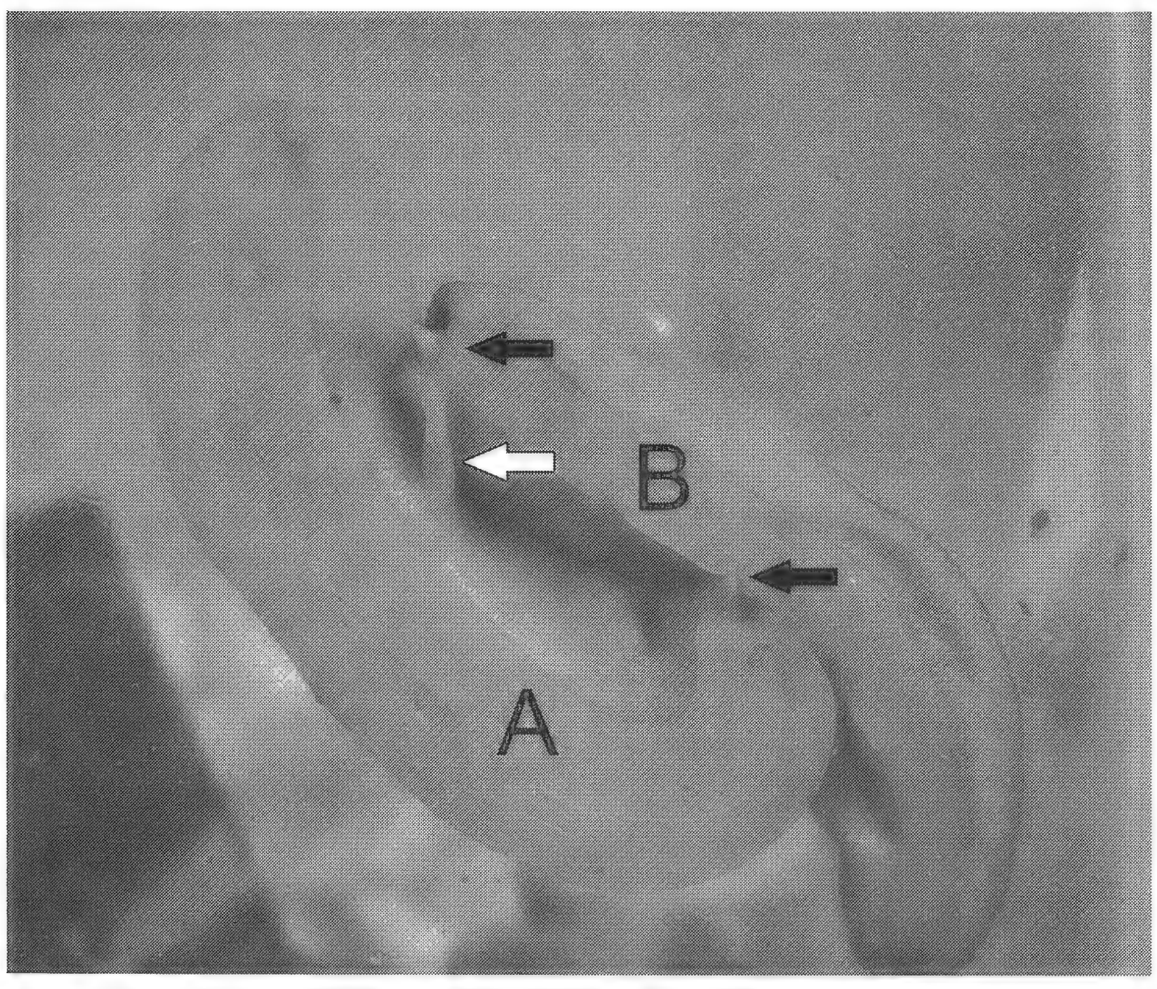

Figure 4. Slugs *in copula* on the side of a glass aquarium. The penis and penial papilla of slug B are clearly visible, while the penis of slug A is partly hidden. Black arrow = penial papilla, white arrow = penis.

During copulation, the penis contracted rhythmically, increasing and decreasing in diameter. The end of copulation was marked by the withdrawal of the penes. In 39% ($N = 28$) of the cases where penis withdrawal was visible, the event was simultaneous. After copulation, individuals immediately began to feed (35%) or remained motionless (65%) for up to three minutes. Individuals mated more than once per night. A pair would cease mating, remain motionless for a time and then copulate again but without the courtship stage, or one or both of the pair would mate with other slugs.

The formation of a triplet mating ring is as follows. Triplets were initiated when an interloper moved between a mating pair. On two occasions, an interloper pushed its way between a mating pair and then lay motionless between them. After a while, each of the mating individuals retracted its penis. The three slugs then commenced circling and brushing before copulating. On another occasion, a mating pair (A and B) was interrupted by an interloper that rubbed its body against 'B' in close proximity to individual B's female genital pore. With its penis darting out repeatedly, the interloper continued to push against the mating slugs. After 20 minutes, individual A began to withdraw its penis. The interloper then pushed the mating pair farther apart and began brushing against the body of individual A, at which time individual B began to withdraw its penis. With copulation between the original mating pair now over, the three individuals started to circle while maintaining body contact. Seven minutes later a mating triplet formed. The sequence of events in triplet mating is summarized in Figure 5. A slug approaches a mating pair and pushes underneath them stretching the penis of the mating individuals (Figs. 5A–D). The mating pair separates (Fig. 5E) and two slugs compete for the genital pore of the third slug; circling begins (Fig. 5F). Circles decrease in size and copulation occurs (Fig. 5H). Triplet mating and interlopers (Fig. 6) were also observed in the field.

One hundred slugs (50 mating couplets) were collected from the wild during the study. The length of these slugs ranged between 55 mm and 120 mm. There was a highly significant positive correlation between the lengths of mating partners ($R^2 = 0.9656$, $P = < 0.001$) (Fig. 7), as mating pairs were always comprised of individuals of similar length. The mean difference in length between members of a pair was 3.8 mm (range 0.7–13).

DISCUSSION

Mating in *Veronicella sloanii* was comprised of the two phases, courtship and copulation. The duration of courtship in *V. sloanii* is short, often less than two minutes. Rueda

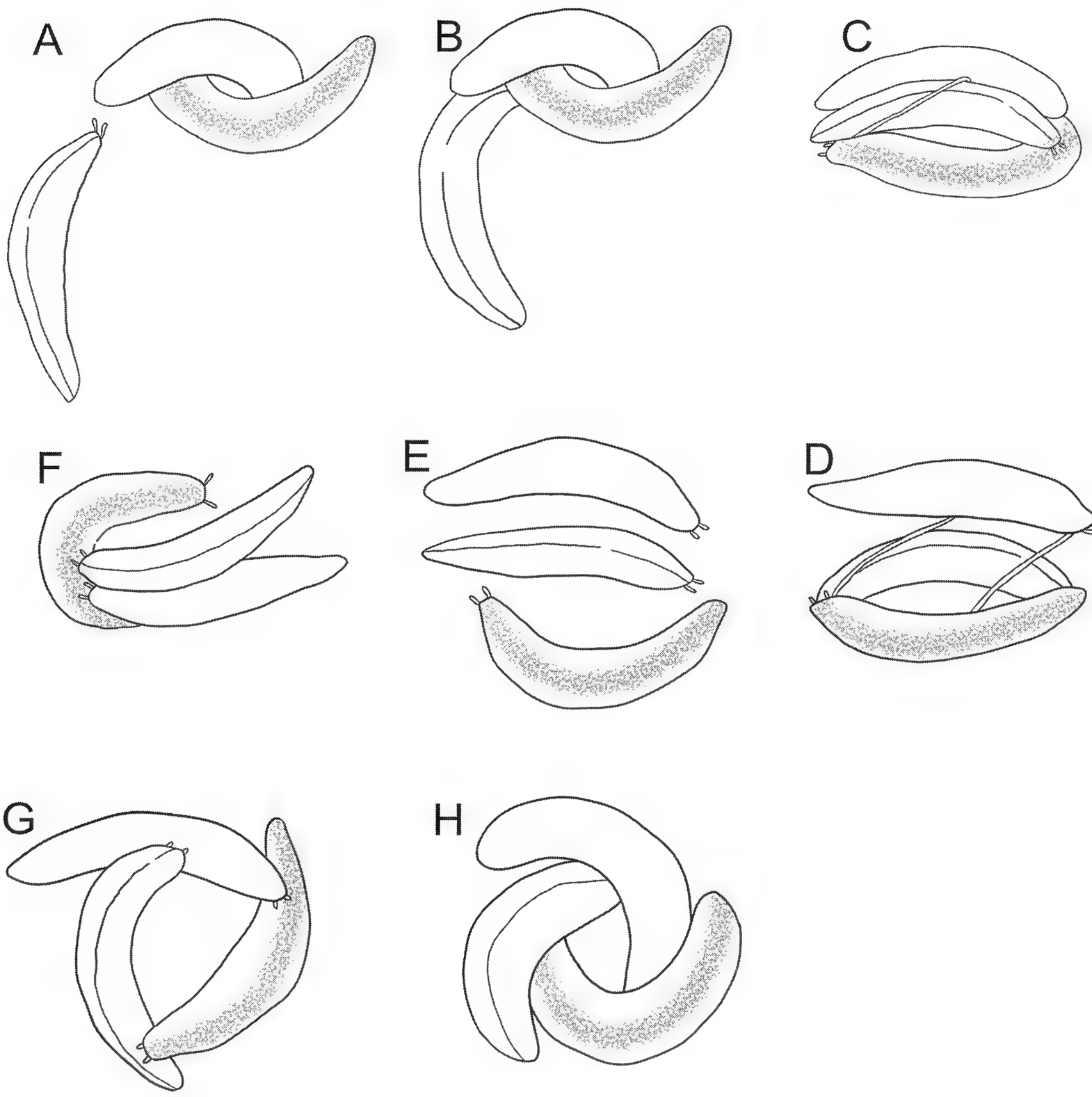

Figure 5. Stages in the formation of a mating ring in *Veronicella sloanii*. **A,** Interloper approaches a mating pair; **B**, contact is made. **C,** stretched penis of one individual visible. **D,** withdrawal of the penes; **E,** complete separation of original mating pair; **F,** two slugs vie for the female genital pore of one individual; **G,** alignment of genitalia; **H,** intromission.

(1989) noted that there was no courtship in *Sarasinula plebeia*, while for *Phyllocaulis boraceiensis*, Thomé (1968) reported that courtship lasted just under an hour. The duration of courtship was not mentioned for the other veronicellids discussed here. In general, courtship in stylommatophoran slugs lasted longer than that observed in *V. sloanii*, ranging from several minutes in *Limax flavus* Linnaeus, 1758 and *Milax gagates* (Draparnaud, 1801) (Karlin and Bacon 1961) to several hours in *Deroceras* spp. Rafinesque, 1820 (Karlin and Bacon 1961, Reise 1995, Riese 2007, Reise *et al.* 2007).

Courtship behaviors seen in *Veronicella sloanii*, including rubbing of the notum with the tentacles, brushing of the body, circling, and swelling of the female genital pore are reported for *Phyllocaulis soleiformis* (Thomé 1968), and lifting of the hyponotum for *Veronicella tenax* (Rivero 1946). Circling of individuals during courtship also occurs in milacids (Forcardi and Quattrini 1972, Barker 1999), agriolimacids (Karlin and Bacon 1961, Reise 1995, Reise 2007, Reise *et al.* 2007) and limacids (Karlin and Bacon 1961, Langlois 1965). In the agriolimacids, ariolimacids and the limacids, as in *Veronicella sloanii*, circling was accompanied by body contact (Karlin and Bacon 1961, Reise 1995, Leonard *et al.* 2002).

Couplet mating in *Veronicella sloanii* is simultaneously reciprocal. Such simultaneously reciprocal copulation has been reported for *Diplosolenodes* (Gerhardt 1937), *Phyllocaulis soleiformis* (Thomé 1968), *Phyllocaulis boraceiensis* (Thomé *et al.* 1999), *Belocaulis angustipes* (Rueda 1989, Bill Frank pers. comm.) and *Sarasinula plebeia* (Rueda 1989). In *P. soleiformis* and *S. plebeia*, the initial alignment of mating individuals was T-shaped, and at this time only one of the pair achieved intromission (Thomé 1968, Rueda 1989). Reciprocity occurred sometime later, approximately after four minutes in *Sarasinula plebeia* (Rueda 1989). Rueda (1989) also noted instances of unilateral mating in *Sarasinula plebeia* that never transitioned into reciprocal mating and an instance of sequential reciprocity, where an individual mated first as a male and then as a female in a subsequent mating a short while later. Only the T-shape mating arrangement has been reported for *Veronicella tenax* (Rivero 1946) and for *Sarasinula linguaeformis* (Mansur and Thomé 1994). This T-shaped arrangement of mating individuals is similar to that seen when *V. sloanii* pushes its head under the body of another slug during initial contact (Fig. 2) but, at no time in *V. sloanii* does intromission occur in this position. Intromission in *V. sloanii* occurs only after the male and female genital pores of the partners are opposed.

Veronicella sloanii also mates with more than one partner, forming a ring comprised of three individuals. Mating rings were also seen in the field and are, therefore, not an artifact of mating in the close confines of an aquarium. This is the first record of a mating ring in a terrestrial slug. In terrestrial snails and slugs, copulation generally occurs between two individuals only, but in some marine and freshwater molluscs mating chains of three or more individuals have been reported. Marine species exhibiting linear chain copulation include opistobranchs such as *Aplysia* Linnaeus, 1767 (Susswein *et al.* 1984, Zaferes *et al.* 1988, Pennings 1991, Yusa

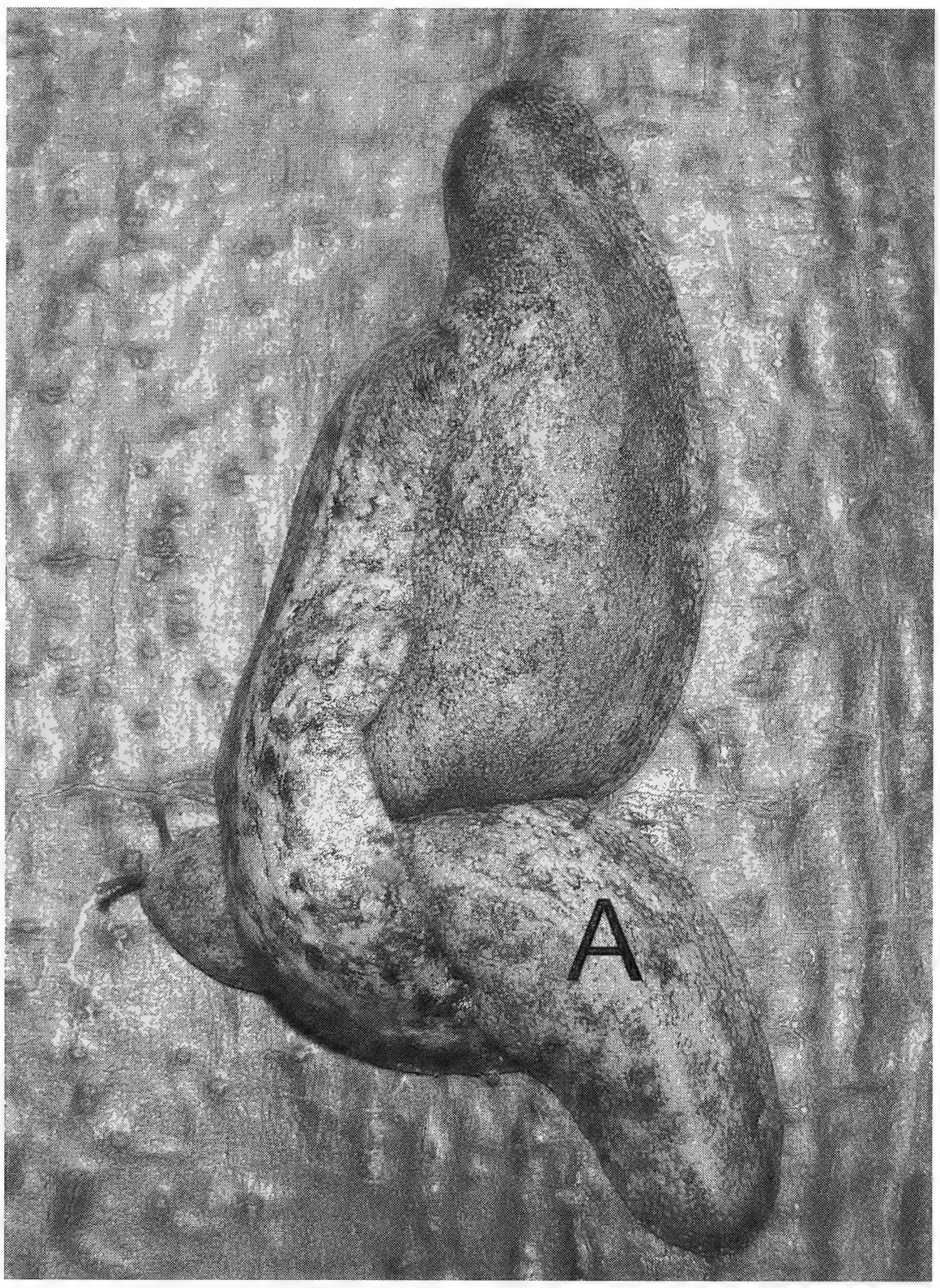

Figure 6. Mating pair and interloper on a tree trunk in Mount Wilton gully, Barbados. **A**, Interloper.

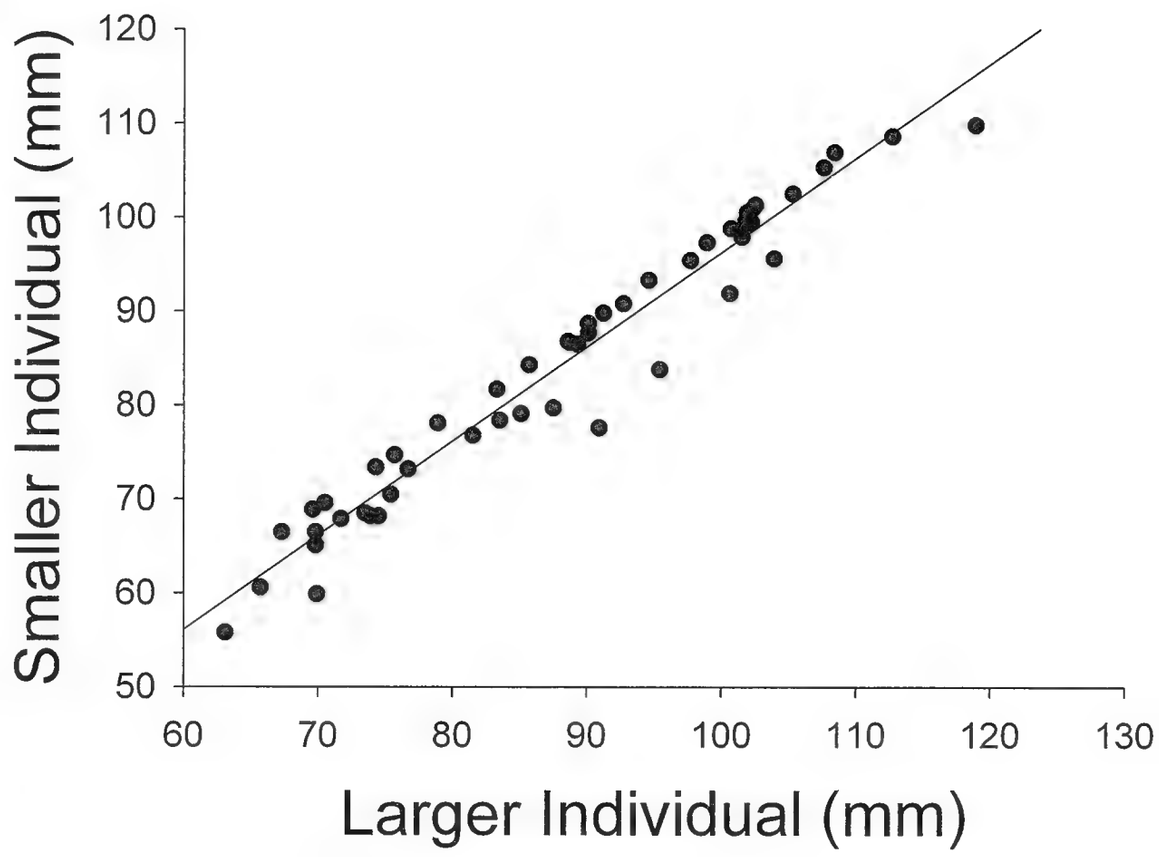

Figure 7. Relationship between lengths of individuals in mating pairs. ($R^2 = 0.9565$, $P < 0.001$).

1996, Angeloni *et al.* 1999, Angeloni *et al.* 2003), *Chelidonura* Adams, 1850 (Anthes and Michiels 2007), *Bulla gouldiana* Pilsbry, 1895 and *Navanax inermis* Cooper, 1863, although, in the latter species, this behavior does not commonly occur in the wild (Leonard and Lukowiak 1985). In addition to the linear chains, some species such as *Navanax inermis* (Leonard and Lukowiak 1985) and *Chelidonura* (Anthes and Michiels 2007) form closed chains while in *Aplysia fasciata* Poiret, 1789 branched chains have been reported (Susswein *et al.* 1984). In the freshwater basommatophorans, some planorbids (Trigwell and Dussart 1998), ancylids (Geldiay 1956), lymnaeids (Crabb 1927) and physids (Duncan 1959) also form linear chains. In a terrestrial pulmonate, *Sarasinula plebeia*, simultaneous multi-partner mating was observed by Rueda (1989), where the mating slugs formed a chain that on one occasion involved five individuals.

Mating chains form, as exemplified by *Navanax inermis* (Leonard and Lukowiak 1987), when an individual initiates copulation, in either the male or the female role, with a member of a pair already *in copula*. In linear chains the individual at the head of the chain acts as a female and the individual at the rear of the chain acts as a male; all other individuals act as both male and female simultaneously (Valdés *et al.* 2010). In closed chains, all individuals act in both the male and female role simultaneously. The formation of mating chains is, thus, dependent on the ability of individuals to function in both the male and female role at the same time. In *Sarasinula plebeia, Navanax inermis,* aplysiids and the fresh water species, a 'free' genital pore is available for intromission, while in *Veronicella sloanii* an individual has to displace one of the mating individuals from a female genital pore in order to form a mating ring.

In *Veronicella sloanii* an accessory organ, the penial papilla, is used during mating to stroke the hyponotum and or the foot of its mating partner intermittently during copulation. Similar placement of this gland during copulation is reported for *Leidyula floridana* (Rueda 1989). In contrast, during copulation in *Belocaulis angustipes*, the penial gland made contact with the notum rather than the hyponotum of the mate and the contact was more forceful, often appearing to cause bruising (Rueda 1989). Use of this gland was not observed in the other veronicellid slugs. The function of the penial papilla is unknown. We propose here that one of its roles is to maintain copulation, based on the observation that during couplet mating in *V. sloanii*, withdrawal of the penis occurs and copulation ends when stroking of one of the partners ceases. Whether it also functions to influence the motility of the female reproductive tract (Koene and Chase 1998) or enhance sperm survival (Chase and Blanchard 2006, Landolfa *et al.* 2001) is a subject for further study. Accessory organs are also used during courtship and copulation in agriolimacids

(Karlin and Bacon 1961, Reise 1995, Reise 2007, Reise *et al.* 2007) and milacids (Focardi and Quattrini 1972, Barker 1999).

The duration of copulation in *Veronicella sloanii* is comparable to that reported for other veronicellids, 30 minutes in *Sarasinula plebeia* (Rueda 1989), one hour in *Phylocaulis soleiformis* (Thomé 1968) and six hours in species of *Leidyula* Baker 1925 (Rueda 1989), but never as long as the 8 to 40 hours reported for *Milax gagates* (Forcardi and Quatrini 1972, Barker 1999). In the current study, two hours was the longest *V. sloanii* remained *in copula*; however copulation lasting up to 5 hours can occur (personal observation).

In *Veronicella sloanii*, a lengthy copulation period potentially exposes the mating individuals to the risk of predation and desiccation, as in their natural environment, slugs mate in exposed areas, both at night and in the daytime. However, prolonged copulation has been shown to reduce sperm competition (Parker 1970, Andrés and Rivera 2000, Harari et. *al.* 2003) through mate guarding. Mate guarding reduces the probability of the female mating with rival males (Parker 1970, Simmons and Siva-Jothy 1998, Andrés and Rivera 2000) and in some species increases the chances of the guarding male's sperm fertilizing the female's eggs (Simmons and Siva-Jothy 1998, Wang *et al.* 2008). Prolonged copulation has also been shown to decrease sperm competition through sperm displacement (Parker 1970, Oglesby *et al.* 1981, Burela and Martín 2011) and sperm loading (Parker 1970, García-González and Gomendio 2004). However, Burela and Martín (2011) found in *Pomacea caniculata* (Lamarck, 1822), that the primary function of the long copulation was to transfer the large ejaculate needed to fertilize its eggs. Thus, while prolonged copulation may reduce sperm competition in *V. sloanii*, as it restricts access to a female genital pore, the possibility that a lengthy copulation may be needed to transfer a large quantity of sperm cannot be ruled out.

In our study, we observed that once a mating event is initiated, other slugs will move towards the mating pair and begin to copulate with no obvious interaction with the original mating pair. This phenomenon was also reported in *Sarasinula plebeia* (Rueda 1989). Mating aggregates composed of both mating and non-mating individuals, similar to those seen in *Veronicella sloanii*, have been observed in *Sarasinula plebeia* (Rueda 1989), *Veronicella cubensis* (Pfeiffer, 1840) (David Robinson, pers. comm.) and *Belocaulis angustipes* (Bill Frank, pers. comm.). Rueda (1989) suggested that a volatile pheromone may be involved in the mating aggregates observed in *Sarasinula plebeia.* Pheromones have been identified as a causative agent in the mating aggregations of aplysiids (Painter *et al.* 1998, Cummins *et al.* 2004) and while no pheromone has been identified as the cause of mating aggregations in terrestrial snails (Croll 1983), its presence has been implied. While secretions from the head-wart of *Euhadra peliomphala* Pfeiffer, 1850 were reported to be a stimulant rather than an attractant (Takeda and Tsuruoka 1979) Falkner (1993) linked the activity of head-warts during courtship in bradybaenids and helicids to the attraction of conspecifics to a 'courting' individual. Trail following linked to mate location has been reported in four genera of land snail (Ng *et al.* 2013). Whether it is airborne chemicals or those that have been deposited in mucous trails that underlie the formation of mating aggregates in *V. sloanii* cannot be resolved at this time from the data available.

Aggressive behavior was observed in *Veronicella sloanii.* However behaviors such as biting and apophallation seen in *Ariolimax* Mörch, 1859(Heath 1916, Leonard *et al.* 2002), and slime feeding in *Deroceras reticulatum* (O. F. Müller, 1774) and the limacids (Karlin and Bacon 1961) do not occur in *V. sloanii.*

During the study, it was found that individuals of *Veronicella sloanii* in a mating pair were of similar lengths, which is an example of size-assortative mating. Size-assortative mating is known to occur in other hermaphroditic molluscs such as the opisthobranch, *Hypselodoris* (= *Chromodoris*) *zebra* (Heilprin, 1889) (Crozier 1917, 1918), the limpets, *Siphonaria capensis* Quoy and Gaimard, 1833 (Chase *et al.* 2010) and *S. gigas* Sowerby 1825 (Levings and Garrity 1986 cited in Chase *et al.* 2010) and older individuals of the giant African snail, *Achatina fulica* Bowdich, 1822 (Tomiyama 1996). A trend towards size-assortative mating has been reported in *Aplysia vaccaria* (Angeloni and Bradbury 1999), *A. californica* Cooper, 1863 (Angeloni *et al.* 2003) and *Helix pomatia* (Baur 1992). Size-assortative mating was not observed in *Succinea putris* (Linnaeus, 1758) (Jordaens *et al.* 2005) or in *Arianta arbustorum* Linnaeus, 1758 (Baur 1992). Three hypotheses have been put forward to explain size-assortative mating seen in organisms: mate choice, mate availability and mating constraints (McLain 1984, Crespi 1989, Harari *et al.* 1999, Chase *et al.* 2010). In *V. sloanii*, it is clear that mate choice does exist as not all courtship events initiated by the slugs in our study resulted in copulation and we recorded the existence of a highly significant size-assortative mating pattern. This could result if both individuals seeking to mate have a preference for mating with a large animal as this will lead to large individuals mating with other large individuals leaving the smaller animals to choose similar sized individuals. The possibility of size-assortative mating due to mate availability because of spatial or temporal distributions of the species cannot be excluded, but this would require that similar sized animals are unequally distributed in time and space, something that we have not observed to be true for *V. sloanii.* Physical constraints may play an important role in explaining the very strong size-assortative mating observed in *V. sloanii* because of the configuration the animals have to assume during copulation. The genitalia must be aligned and the penial papilla

must be able to make contact with the body of its mating partner in order to maintain copulation. Crozier (1918) proposed that the inability to align the genitalia in *Hypselodoris zebra* could explain the size-assortative mating seen in this species. In *H. zebra*, this alignment need occur only at one location in the mating pair but, in *V. sloanii* this alignment of genitalia must take place at two locations (Fig. 4). This restricts the range of positional adjustments that could be made to align the genitalia in individuals that vary markedly in length. That a combination of causes, rather than any one may explain completely size-assortative mating in a given species is discussed by (Harari *et al.* 1999) and this may be the case in *V. sloanii*. Regardless of the underlying cause or causes of the size-assortative mating pattern seen in *V. sloanii*, assessment of the size of a potential mate should be very important in this species. Assessment of size may occur during 'body brushing', a suggestion also made by Riese *et al.* (2007) for *Deroceras gorgonium* Wiktor, Vardinoy-annis and Mylonas, 1994. A disparity in size could form the basis of rejection of a potential mate.

We conclude that mating in *Veronicella sloanii* is similar to that of other veronicellids and that some of its behaviors resemble those of stylommatophoran slugs. The observation of multi-partner mating rings in this species adds a new dimension to what is already a varied suite of mating behaviors in veronicellid and stylommatophoran slugs. In addition, the strong size-assortative mating pattern seen in *V. sloanii* has not been reported in other terrestrial gastropods.

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First records of four exotic slugs in Argentina

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Abstract: This paper reports for the first time the occurrence of four exotic terrestrial slug species in Argentina: *Lehmannia valentiana* (Férussac, 1823) (Limacidae), *Deroceras invadens* Reise *et al.* 2011 (Agriolimacidae), *Arion intermedius* Normand, 1852 (Arionidae) and *Meghimatium pictum* (Stolyczka, 1873) (Philomycidae). The study is based on specimens deposited in museums in Argentina. Both the morphologic characteristics and the mitochondrial cytochrome oxidase I gene sequences were used to identify the exotic species. Phylogenetic analyses were also carried out in order to explore the location of their origins. *Lehmannia valentiana* had the oldest records and has been widely distributed in Argentina. *Deroceras invadens* and *A. intermedius* were found to be restricted to the southern portion of the country. *Meghimatium pictum* was recorded in the northwest and northeast Argentina, and the DNA sequences analyzed from this species were more closely related to specimens from the west of the Strait of Taiwan. A determination of the origin of the other species was impossible because either the sequences analyzed grouped with samples from different geographical origins or only few sequences were available for comparison. In view of the invasive potential of these slug species, the present work provides new and potentially useful DNA sequence data obtained from morphologically-confirmed specimens. Information provided from these analyses should assist in making a rapid identification of these exotic slugs by nonspecialists and governmental authorities who are responsible for managing and controlling the presence of exotic species.

Key words: *Lehmannia valentiana, Deroceras invadens, Arion intermedius, Meghimatium pictum,* molecular phylogenetic analyses

Biologic invasions, together with climate change and habitat fragmentation, constitute one of the most serious threats affecting the maintenance of global biodiversity (Vitousek *et al.* 1996, Nentwig 2007). Bioinvasions have mostly caused disruptive effects on native species, alteration of ecological processes, changes in natural systems, and economic and human-health problems (Mooney *et al.* 2005). In South America, for example, certain invasive mollusk species – *e.g.,* the continental bivalve *Limnoperna fortunei* (Dunker, 1857), the marine gastropod *Rapana venosa* (Valenciennes, 1846) and the terrestrial snail *Achatina* (*Lissachatina*) *fulica* Bowdich, 1822 – have been reported to reduce biodiversity (Darrigran and Damborenea 2006, Giberto *et al.* 2006, Thiengo *et al.* 2007). Some introductions have been deliberate (*e.g., A. fulica*), while others were unintentional (*e.g., L. fortunei*), with the latter being the most common case in point (Cowie and Robinson 2003).

Twenty-three exotic terrestrial gastropod species have been reported in Argentina (Parent and Miquel 1999, Miquel *et al.* 2007, Rumi *et al.* 2010, Gutiérrez Gregoric *et al.* 2011). Six of these species are slugs: *Limacus flavus* Linnaeus, 1758, *Limax maximus* Linnaeus, 1758, *Deroceras agreste* (Linnaeus, 1758), *Deroceras laeve* (Müller, 1774), *Deroceras reticulatum* (Müller, 1774), and *Milax gagates* (Drapaurnaud, 1801) (Rumi *et al.* 2010). Because of the high intraspecific morphologic variability in slugs, molecular studies have proven to be extremely useful for species identification (McDonnell *et al.* 2008, 2011). Phylogenetic studies conducted on several species have, moreover, enabled a more complete understanding of the history of the invasions and an establishment of the origins of the invaders along with the pathways of their introduction (*e.g.,* Facon *et al.* 2003). These molecular studies, combined with anatomical taxonomic identification and an understanding of those pathways, constitute crucial information for enabling governmental entities to adjust their strategies for controlling invading species (Cowie and Robinson 2003, Lizarralde *et al.* 2008).

The aim of this report is to document for the first time in Argentina the presence of four alien terrestrial slugs from

the families Limacidae, Agriolimacidae, Arionidae and Philomycidae, on the basis of morphologic and molecular-genetic identifications and, wherever possible, to make inferences about the origins of those species and the possible consequences of their introductions.

MATERIALS AND METHODS

Specimen collections and morphology-based identifications

Malacological collections from the Museo de La Plata (MLP), the Museo Argentino de Ciencias Naturales (MACN-In), the Instituto Fundación Miguel Lillo (IFML), and the United States Department of Agriculture (USDA) were surveyed (Table 1). The specimens chosen (Limacidae $N = 43$; Agriolimacidae $N = 62$; Arionidae $N = 14$; Philomycidae $N = 12$) were dissected under a stereomicroscope and identified mainly on the basis of the reproductive system and/or the external morphology. Adult specimens, properly relaxed, were measured to establish a length range.

DNA extraction, polymerase chain reaction (PCR) amplification, and genetic sequencing

Total DNA was extracted from 2 mm^3 samples from the foot of each dissected slug. The tissue was rinsed in distilled

Table 1. Material examined from slugs from Argentina. **IFML,** Instituto Fundación Miguel Lillo; **MACN-In,** Museo Argentino de Ciencias Naturales; **MLP,** Museo de La Plata; ***N,*** number of specimens; **USDA,** United States Department of Agriculture.

Family	Province	Site	Date	Collection	*N*	Coordinates
Limacidae	Tucumán	San Miquel	1962	IFML 604	28	26°49′S, 65°12′W
	Chubut	Lago Puelo Nat. Park	2003	IFML 14431	4	42°05′S, 71°36′W
	Buenos Aires	Sierras Bayas	1924	MACN-In 14602	5	36°56′S, 60°09′W
		La Plata	2012	MLP 13636	8	34°55′S, 57°55′W
	Río Negro	El Bolsón	2004	MACN-In 36158	1	51°58′S, 71°32′W
	Neuquén	Neuquén	2004	MACN-In 36162	1	38°56′S, 68°03′W
Agriolimacidae	Río Negro	El Bolsón	2004	MACN-In 36157 MACN-In 36158/1	2	51°58′S, 71°32′W
		Dina Huapi	2004	MACN-In 36167 MACN-In 36168/1	4	41°05′S, 71°10′W
		San Carlos de Bariloche	2004	MACN-In 36173 MACN-In 36179–80 MACN-In 36182–83 MACN-In 36186/2 MACN-In 36188–92	33	41°08′S, 71°18′W
		Cerro Otto	2004	MACN-In 36177	4	41°08′S, 71°19′W
		Nahuel Malal	2004	MACN-In 36176	2	41°06′S, 71°26′W
		Manantial	2004	MACN-In 36181/1	2	40°40′S, 71°37′W
	Neuquén	Neuquén	2004	MACN-In 36162	1	38°56′S, 68°03′W
		Isla Victoria	2004	MACN-In 36164	12	40°58′S, 71°31′W
		Villa La Angostura	2004	MACN-In 36169	4	40°45′S, 71°38′W
Arionidae	Chubut	Los Alerces Nat. Park	2010	MLP 13405 IFML 15569	12	42°36′S, 71°38′W
		Los Alerces Nat. Park	2003	MLP 13404 IFML 15463	5	42°44′S, 71°44′W
		Lago Puelo Nat. Park	2003	IFML 15449 IFML 15566	13	42°05′S, 71°36′W
		Lago Puelo Nat. Park	2004	MACN-In 36184	2	42°05′S, 71°37′W
	Río Negro	Guillelmo lake	2010	IFML 15568 A	3	41°21′S, 71°29′W
		San Carlos de Bariloche	2004	MACN-In 36170 MACN-In 36186/1	4	41°05′S, 71°27′W
Philomycidae	Misiones	Iguazú Nat. Park	2009	MLP 13402	3	25°41′S, 54°27′W
		Tabay Fall	2009	MLP 13403	5	27°00′S, 55°11′W
		Puerto Iguazú	2011	IFMLP 15570 A	1	25°35′S, 54°34′W
	Tucumán	San Miguel	1999	IFML 15571 A	1	26°48′S, 65°17′W
	Brazil	Foz do Iguaçu	2011	USDA 110442	2	25°34′S, 54°34′W

water, ground in 100 mM EDTA and 20 mM Tris, and digested overnight in CTAB buffer containing proteinase K. DNA was purified by a threefold extraction with chloroform-isoamyl alcohol (24:1) followed by precipitation with isopropanol. The DNA was then resuspended in TE buffer. A 655 bp fragment of the gene encoding the mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified by means of the primers of Folmer *et al.* (1994).

Amplification was performed in a final volume of 50 µl containing: 50–100 ng of template DNA, 0.1 µM of each primer, 1X PCR buffer, 50 µM dNTPs, 2.5 mM $MgCl_2$, and 1.2 U Platinum *Taq* polymerase (Invitrogen, Brazil). The thermocycling sequence consisted of 3 min at 94 °C; 5 cycles of 30 s at 94 °C; 40 s at 45 °C; 1 min at 72 °C; followed by 35 cycles of 30 s at 94 °C; 40 s at 51 °C; 1 min at 72 °C; with a final extension for 10 min at 72 °C. After purification of the PCR products by electrophoresis in 1.5% (w/v) agarose gels through the use of a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, California), both DNA strands were sequenced (Macrogen Inc., Seoul, Korea). The resulting sequences were trimmed to remove the primers, and the consensus sequences of the individuals were compared to reference sequences in GenBank through the use of the BLASTN algorithm (Altschul *et al.* 1990) to identify similarities.

Phylogenetic analyses

Phylogenetic analyses were conducted in order to confirm the morphology-based identification of the specimens found in Argentina and to make inferences on the source location whenever that identification was possible. For the Agriolimacidae we used the COI sequences of almost all the species represented in Reise *et al.* (2011) (Table 2). For the Arionidae, we analyzed the COI sequences available in GenBank mainly for the *Arion* Férussac, 1819 species, those being reported in the literature as either invasive or potentially invasive (McDonnell *et al.* 2009, Thomas *et al.* 2010) (Table 3). For the Philomycidae, we conducted the phylogenetic analyses with the COI sequences for only *Meghimatium pictum* (Stolyczka, 1873) since we counted on a large number of available sequences for that species from various locations

Table 2. Information on the specimens used in the phylogenetic reconstruction of *Deroceras* species. *GenBank unpublished sequences: the sequence author and submission year are indicated.

Taxon	GenBank #	Country	Reference
Outgroup			
Arion distinctus Mabille, 1868	EF128218	Taiwan	Tsai and Wu 2008
Arion rufus (Linnaeus, 1758)	FJ481178	-	Tsai 2008*
Pallifera dorsalis (A. Binney, 1842)	FJ896618	U.S.A.	Tsai *et al.* 2011
Philomycus carolinianus (Bosc, 1802)	EF128221	U.S.A.	Tsai *et al.* 2011
Megapallifera ragsdalei (Webb, 1950)	EF128220	U.S.A.	Tsai and Wu 2008
Ingroup			
Deroceras golcheri (Altena, 1962)	JN248291–293	Malta	Reise *et al.* 2011
Deroceras invadens	FJ358222	South Africa	Reise *et al.* 2011
	JN248295	Germany	Reise *et al.* 2011
	JN248296	United Kingdom	Reise *et al.* 2011
	JN248297	Canada	Reise *et al.* 2011
	JN248298–300	Italy	Reise *et al.* 2011
	JN248301–302	Germany	Reise *et al.* 2011
	JN248303	United Kingdom	Reise *et al.* 2011
	JN248314	France	Reise *et al.* 2011
	JN248315	U.S.A.	Reise *et al.* 2011
	JQ743070	Argentina	This work
Deroceras laeve	AF239733	U.S.A.	Reise *et al.* 2011
	EF128217	Taiwan	Tsai and Wu 2008
	HM584699	-	Reise *et al.* 2011
Deroceras panormitanum	JN248304–306	Italy	Reise *et al.* 2011
	JN248307–311	Malta	Reise *et al.* 2011
	JN248312–313	Italy	Reise *et al.* 2011
Deroceras reticulatum	AF239734	U.S.A.	Reise *et al.* 2011
	AM259702–703	United Kingdom	Reise *et al.* 2011
	FJ481179	-	Reise *et al.* 2011

Table 3. Information on the specimens used in the phylogenetic reconstruction of *Arion* species. *GenBank unpublished sequences: the sequence author and submission year are indicated.

Taxon	GenBank #	Country	Reference
Outgroup			
Deroceras leave	EF128217	Taiwan	Tsai and Wu 2008
Deroceras reticulatum	FJ481179	-	Reise *et al.* 2011
Limacus flavus	FJ481181	-	Tsai 2008*
Megapallifera ragsdalei	EF128220	U.S.A.	Tsai and Wu 2008
Philomycus carolinianus	EF128221	U.S.A.	Tsai and Wu 2008
Ingroup			
Arion circumscriptus Johnston, 1828	AY094600	Lithuania	Soroka 2002*
	AY987872	Ireland	Davison *et al.* 2009
	AY987873	Belgium	Davison *et al.* 2009
	DQ647392	Lithuania	Soroka 2006*
Arion distinctus	AY094599	Poland	Soroka and Skujienė 2011
	AY987874	Belgium	Pinceel *et al.* 2005*
	AY987876	Belgium	Pinceel *et al.* 2005*
	DQ647393	Poland	Soroka and Skujienė 2011
Arion fasciatus (Nilsson, 1823)	AF239735	U.S.A.	Remigio and Hebert 2003
	AY094598	Lithuania	Soroka 2002*
	AY987877	Germany	Davison *et al.* 2009
	AY987878	Austria	Davison *et al.* 2009
Arion fuscus (Müller, 1774)	AY094597	Lithuania	Soroka and Skujienė 2011
	AY987885	Poland	McDonnell *et al.* 2011
	AY987886	Bulgaria	McDonnell *et al.* 2011
	DQ647391	Lithuania	Soroka 2006*
Arion hortensis Férussac, 1819	AY423670	-	Davison *et al.* 2009
	AY423688	-	Dodd *et al.* 2003*
	AY987889	United Kingdom	Davison *et al.* 2009
	EU382742	U.S.A.	McDonnell *et al.* 2008
Arion intermedius	AM259724	United Kingdom	McClymont 2006*
	AY987891	Belgium	Davison *et al.* 2009
	EU382756	U.S.A.	McDonnell *et al.* 2008
	JQ743069	Argentina	This work
Arion lusitanicus Mabille, 1868	AY987894	Belgium	Davison *et al.* 2009
	EF520640	Poland	Soroka *et al.* 2009
	EF535149	Poland	Soroka *et al.* 2009
	EU734828	Belgium	Soroka and Kałuski 2011
Arion rufus	EF520644– 647	Poland	Soroka *et al.* 2009
Arion owenii Davies, 1979	AY423702	-	Davison *et al.* 2009
	AY423703	-	Dodd *et al.* 2003*
	AY987897	United Kingdom	Pinceel *et al.* 2005*
	AY987898	United Kingdom	Davison *et al.* 2009
Arion silvaticus Lohmander, 1937	AF513018	Lithuania	Góbbeler and Klussmann-Kolb 2010
	AY987917– 918	Belgium	Davison *et al.* 2009
Arion subfuscus (Draparnaud, 1805)	AY987905	Belgium	McDonnell *et al.* 2011
	AY987914	France	McDonnell *et al.* 2011
	AY987916	France	McDonnell *et al.* 2011
	GU249583	U.S.A.	McDonnell *et al.* 2011

cited in Gomes *et al.* (2011) and Tsai *et al.* (2011) (Table 4). Finally, phylogenetic analyses were not possible for the Limacidae because few sequences were available in GenBank, with the molecular identification of this species based only on the BLASTN algorithm.

In all instances, the phylogenetic analyses were carried out as follows: the sequence alignment was performed with the Clustal X 2.0.12 software (Larkin *et al.* 2007), optimized by visual inspection, and edited with a word processor. The total lengths of the matrices analyzed were 534 bp for the Limacidae, 552 bp for the Arionidae, and 654 bp for the Philomycidae. The data were subjected to four different phylogenetic analyses by the methods of Neighbor-Joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). The NJ analysis was conducted with MEGA 5.05 software (Tamura *et al.* 2011) through the use of the maximum-composite-likelihood option for computing evolutionary distances (Tamura *et al.* 2004). The MP analysis was carried out with the PAUP*4.0b10 software (Swofford 2002), through the use of heuristic search, characters equally

Table 4. Information on the specimens used in the phylogenetic reconstruction of *Meghimatium pictum*. **CMS-DPE,** Superintendência de Controle de Endemias do Estado de São Paulo, Brazil; **ESRI-MOL,** Endemic Species Research Institute, Taiwan; **MLP,** Museo de La Plata, Argentina; **MZSP,** Museu de Zoologia da Universidade de São Paulo, Brazil; **USDA,** United States Department of Agriculture, U.S.A. *GenBank unpublished sequences: the sequence author and submission year are indicated.

Codification	GenBank #	Country	Location	Reference
Outgroup				
Arion distinctus	EF128218	Taiwan	Wuling farm, Taichung	Tsai and Wu 2008
Arion rufus	FJ481178	-	-	Tsai 2008*
Deroceras laeve	EF128217	Taiwan	Guansi, Hsinchu	Tsai and Wu 2008
Deroceras reticulatum	FJ481179	-	-	Reise *et al.* 2011
Limacus flavus	FJ481181	-	-	Tsai 2008*
Megapallifera ragsdalei	EF128220	U.S.A.	Seatey, Alaska	Tsai and Wu 2008
Pallifera dorsalis	FJ896618	U.S.A.	-	Tsai *et al.* 2011
Philomycus carolinianus	EF128221	U.S.A.	Carter, Tennesee	Tsai and Wu 2008
Ingroup				
Meghimatium pictum				
ARP1 (MLP 13403/1)	JQ712575	Argentina	Tabay Fall, Misiones	This work
ARP2 (MLP 13403/2)	JQ712574	Argentina	Tabay Fall, Misiones	This work
BRP1 (CMS-DPE-95)	HM233929	Brazil	Ribeirão Pires, São Paulo	Gomes *et al.* 2011
BRP2 (MZSP 93842)	HM233928	Brazil	Curitiba, Paraná	Gomes *et al.* 2011
BRP3 (MZSP 93836)	HM233930	Brazil	Palhoça, Santa Catarina	Gomes *et al.* 2011
BRP4 (USDA 110442)	JQ712572	Brazil	Foz do Iguaçu, Paraná	This work
BRP5 (USDA 110442)	JQ712573	Brazil	Foz do Iguaçu, Paraná	This work
CNP1 (ESRI-MOL-07-0123)	FJ896666	China	Linan, Zhejiang	Tsai *et al.* 2011
CNP2 (MZSP 93847)	HM233931	China	Zhongcun, Guangzhou	Gomes *et al.* 2011
TLP1 (ESRI-MOL-05-0024)	FJ896667	Thailand	Bangkok	Tsai *et al.* 2011
TWP1 (ESRI-MOL-08-0565)	FJ896651	Taiwan	Wushikeng, Taichung	Tsai *et al.* 2011
TWP2 (ESRI-MOL-07-0117)	FJ896652	Taiwan	Yousheng river, Taichung	Tsai *et al.* 2011
TWP3 (ESRI-MOL-08-0264)	FJ896653	Taiwan	Lugu, Nantou	Tsai *et al.* 2011
TWP4 (ESRI-MOL-08-0566)	FJ896654	Taiwan	Wanluan, Pingtung	Tsai *et al.* 2011
TWP5 (ESRI-MOL-08-0138)	FJ896655	Taiwan	Litao, Taitung	Tsai *et al.* 2011
TWP6 (ESRI-MOL-06-0295)	FJ896656	Taiwan	Lanyu, Taitung	Tsai *et al.* 2011
TWP7 (ESRI-MOL-99-0001)	FJ896657	Taiwan	Ruisui, Hualien	Tsai *et al.* 2011
TWP8 (ESRI-MOL-02-0003)	FJ896658	Taiwan	Meifeng farm, Nantou	Tsai *et al.* 2011
TWP9 (ESRI-MOL-08-0567)	FJ896659	Taiwan	Jiufen, Rueifang, Taipei	Tsai *et al.* 2011
TWP10 (ESRI-MOL-01-0002)	FJ896660	Taiwan	Taman, Wulai, Taipei	Tsai *et al.* 2011
TWP11 (ESRI-MOL-07-0121)	FJ896661	Taiwan	Kinhu, Kinmen	Tsai *et al.* 2011
TWP12 (ESRI-MOL-07-0122)	FJ896662	Taiwan	Lieyu	Tsai *et al.* 2011
TWP13 (ESRI-MOL-07-0125)	FJ896663	Taiwan	Beigan, Matsu	Tsai *et al.* 2011
TWP14 (ESRI-MOL-06-0296)	FJ896664	Taiwan	Nangan, Matsu	Tsai *et al.* 2011
TWP15 (ESRI-MOL-07-0124)	FJ896665	Taiwan	Dongyin, Matsu	Tsai *et al.* 2011

weighted, tree bisection and reconnection branch-swapping and 10 random stepwise additions. The ML inference was performed by means of the PhyML program (Guindon and Gascuel 2003) available at the public Phylemon2 webserver (http://phylemon.bioinfo.cipf.es; Sánchez *et al.* 2011). The optimal model of nucleotide substitution was evaluated by the likelihood-ratio test and selected by means of the corrected Akaike Information Criterion with the Jmodeltest 0.1.1 software (Posada 2008). The HKY+I+G (for Limacidae), the TIM2+I+G (for Arionidae), and the GTR+I+G (for Phylomicidae) substitution models were used as evolutionary paradigms. The statistical support for the resulting phylogenies were assessed by bootstrapping with either 1,000 (NJ, MP) or 100 (ML) replicates (Felsenstein 1985). The BI was carried out with the Mr. Bayes 3.1.2 software (Ronquist and Huelsenbeck 2003). Two runs were performed simultaneously with 4 Markov chains that went for 1,000,000 generations, sampling every 100 generations. The first 10,000 generations of each run were discarded as burnin, and the remaining 18,000 trees were used to estimate posterior probabilities. All the trees were edited with the TreeGraph 2 software (Stöver and Müller 2010).

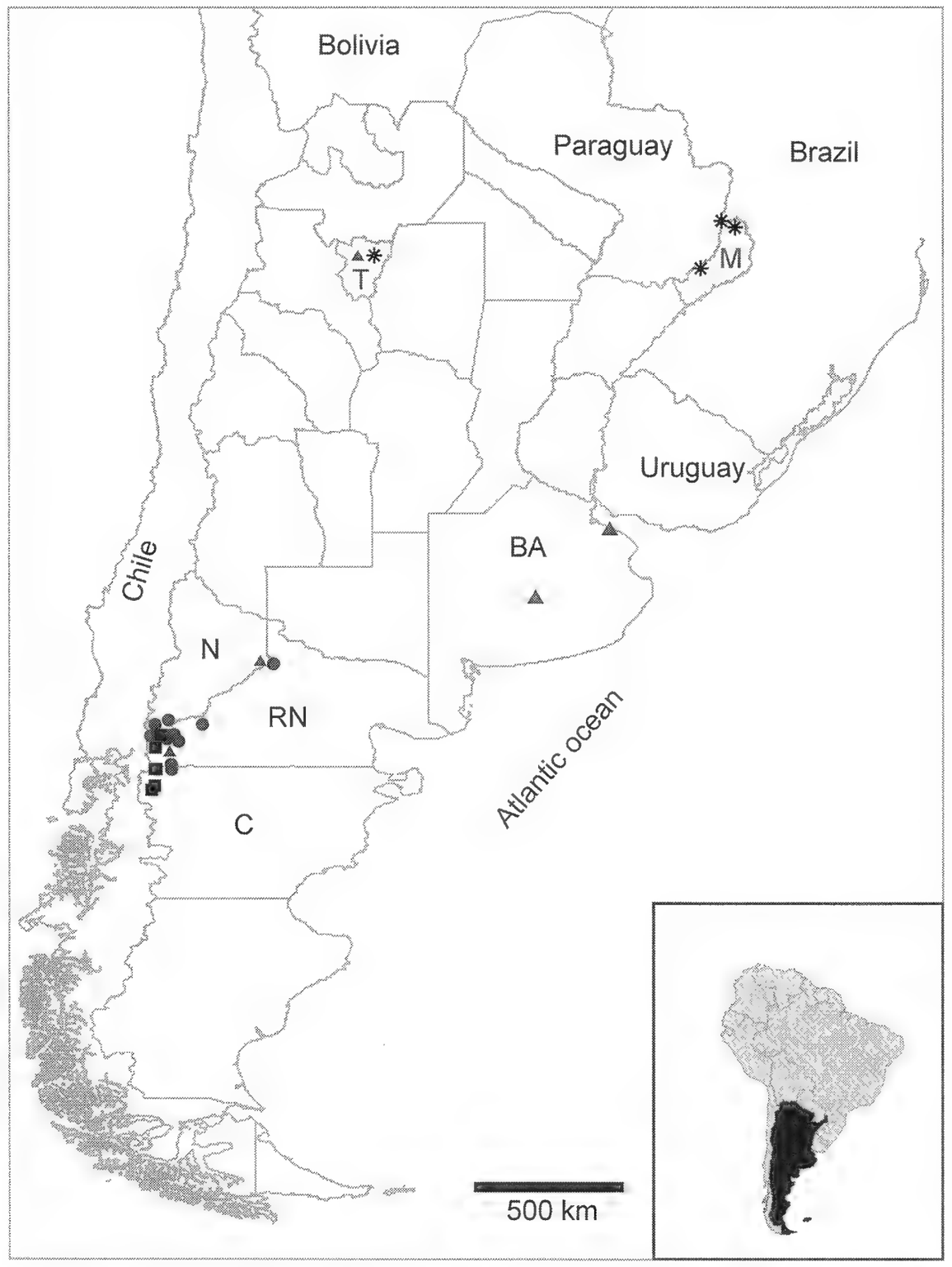

Figure 1. Distribution of new exotic slugs recorded in Argentina. ▲: *Lehmannia valentiana*; ●: *Deroceras invadens*; ■: *Arion intermedius*; *: *Meghimatium pictum.* Provinces: **BA,** Buenos Aires; **C,** Chubut; **M,** Misiones; **N,** Neuquén; **RN,** Río Negro; **T,** Tucumán.

RESULTS

Family LIMACIDAE Lamarck, 1801

The adult specimens had a mean total length of 27.21 mm ($N = 13$; $SD = 5.24$; range = 20.7–39.5 mm), with an ill-defined keel at the hind end. The external morphology and reproductive system of the dissected adult specimens were consistent with descriptions by Castillejos and Garrido (1996) for *Lehmannia valentiana* (Férussac, 1823). Specimens from IFML 14431 were identified only by external morphology. Body color of slugs varied from chestnut or auburn to yellowish gray, with two or three black longitudinal bands on the mantle shield and only two on the rest of the body, finalizing in the posterior end. The central band on the mantle shield was diffuse in some cases. Pneumostome was on the right posterior part of mantle. The specimens had been found in urban and protected zones (*e.g.*, Lago Puelo National Park). *Lehmannia valentiana* has a widespread distribution in Argentina (Fig. 1), and specimens were recorded together with other native and exotic gastropods (*e.g.*, *Deroceras reticulatum* and *Limacus flavus* in La Plata city). The molecular data confirmed the morphology-based identification. The BLASTN search results, with the obtained partial COI sequence as the query sequence (Gen Bank JX117876, 655 bp), showed top-ranking scores and a 99% sequence identity with the only two 554 bp reference COI sequences available in the GenBank nucleotide database (AM259710 and AM259711).

Family AGRIOLIMACIDAE Wagner, 1935

The length of the adult specimens was in general around 12.74 mm ($N = 51$; $SD = 1.38$; range = 9.5–16.4 mm). The external morphology and reproductive system of the dissected specimens ($N = 62$) were consistent with descriptions by Reise *et al.* (2011) for *Deroceras invadens* Reise *et al.*, 2011. This species has been recorded in both urban and protected areas (*e.g.*, Nahuel Huapi and Arrayanes National Parks) (Fig. 1). The identification of the Agriolimacidae specimen analyzed from Argentina based on molecular analysis confirmed its specific identity as *D. invadens*. The trees obtained by different methods indicated a similar topology that was in agreement with Reise *et al.* (2011; Fig. 2 cf. the MP and BI trees). In all instances, this specimen from Argentina belonged to the *D. invadens* clade, which assignment was highly supported (NJ = 100, MP = 98, ML = 100, BI = 1), and was furthermore classified in a subgroup inside this clade (NJ = 87, MP = 93, ML = 84, BI = 0.85) one that included sequences from several parts of the world (*i.e.*, Canada, England, France, Germany, South Africa and U.S.A).

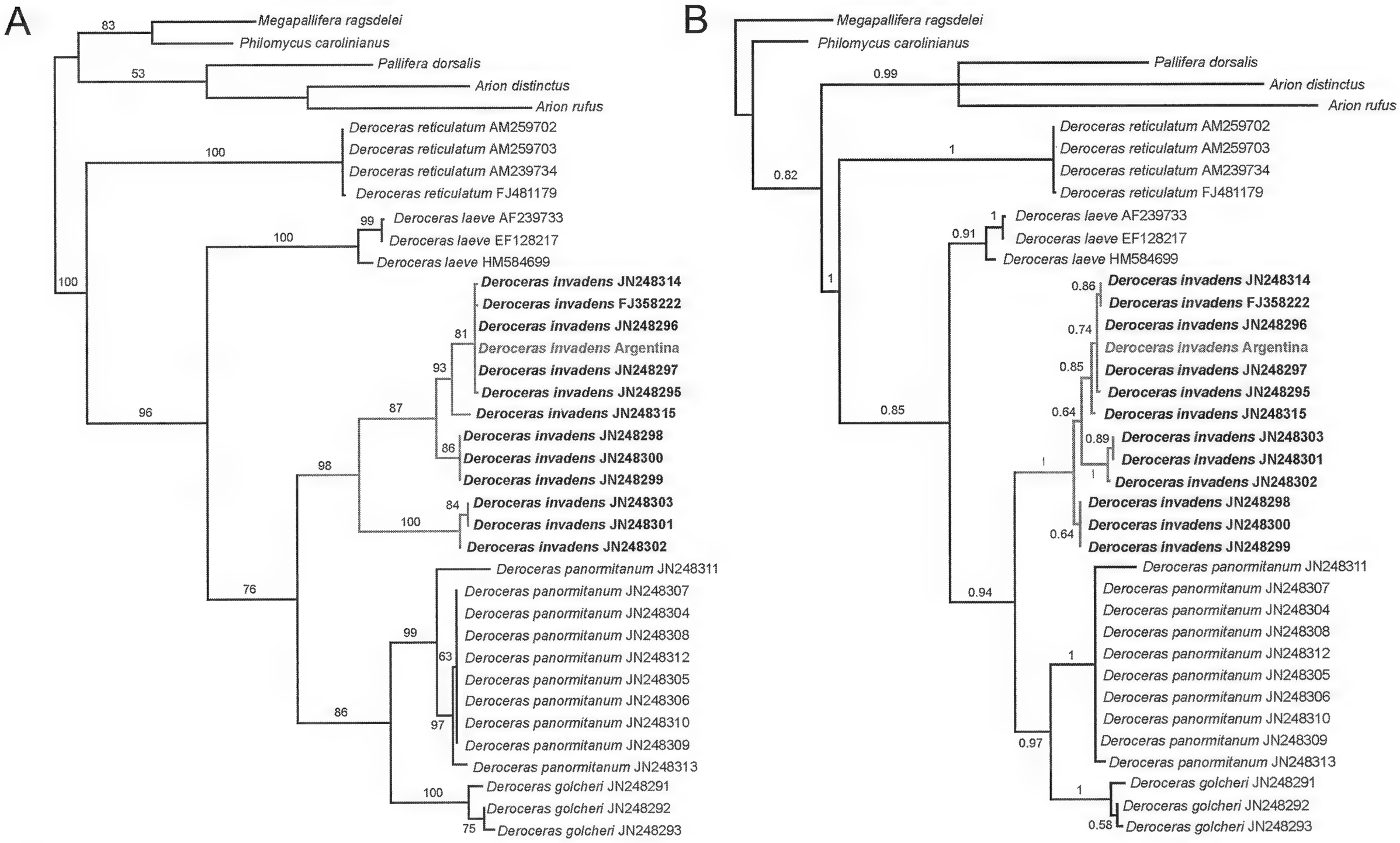

Figure 2. Phylogenetic trees of several *Deroceras* species based on 534 nucleotides of the partial COI gene**. A,** The most parsimonious tree (179 parsimony-informative characters, total length = 519, CI = 0.5934, RI = 0.8252 and, RC = 0.4897). **B,** Bayesian consensus tree. The support values: bootstrap values (MP) and posterior probabilities (BI) are shown on the branches. References to the sequences are given in Table 2.

Family ARIONIDAE Gray, 1840

The specimens had a mean of 10.07 mm of total length ($N = 3$; $SD = 0.74$; range = 9.5–10.9 mm). The external morphology and reproductive system of the dissected adult specimens were consistent with the Barker's descriptions (1999) for *Arion intermedius* Normand, 1852. Specimens from IFML 15449, 15566, 15568 (Table 1) were identified by external morphology. General coloration was golden yellow in most of the collected specimens, without lateral or central bands; ommatophores were dark brown; the mantle was oval with pneumostome on the right side in the antemedial portion; the posterior body section was rounded without keel. The slugs were collected under woodpiles or sheltering under rocks, in synanthropically disturbed locations, such as the service areas of Lago Puelo National Park, and the camping areas in Los Alerces National Park. The identification of the Arionidae specimen (MLP 13405) analyzed by molecular-genetic sequencing confirmed its specific identity as *A. intermedius*. Different phylogenetic analyses gave very similar topological organization for the NJ, MP, and ML trees and minor differences in the BI-tree organization (Fig. 3 cf. the MP and BI trees). The specimen from Argentina was placed within the highly supported *A. intermedius* group (NJ = 100, MP = 100, ML = 100, BI = 1).

Family PHILOMYCIDAE Gray, 1847

Only the specimen from Puerto Iguazú was sexually mature (total length = 50 mm). The morphology of its reproductive system and external morphology coincided with descriptions by Tsai *et al.* (2005) and Gomes *et al.* (2011) for *Meghimatium pictum* (Stolyczka, 1873). Specimens from IFML 15570 A and MLP 13402 (Table 1) were identified by external morphology, which had an opaque beige background color of mantle, with two dark brown to black lateral stripes, and one medial stripe, often lighter than the lateral ones. This species was found in sites ranging from highly anthropically disturbed (in the Misiones and Tucumán provinces) to undisturbed areas (Iguazú National Park; Fig. 1, Table 1). The identification of Philomycidae specimens by DNA-data analysis confirmed their specific identity as *M. pictum* (MLP 13403 and USDA 110442; Table 1). The sequences obtained were identical and larger (655 bp) than previously reported for the species in South America (Gomes *et al.* 2011). All four phylogenetic approaches provided similar results and indicated a topology

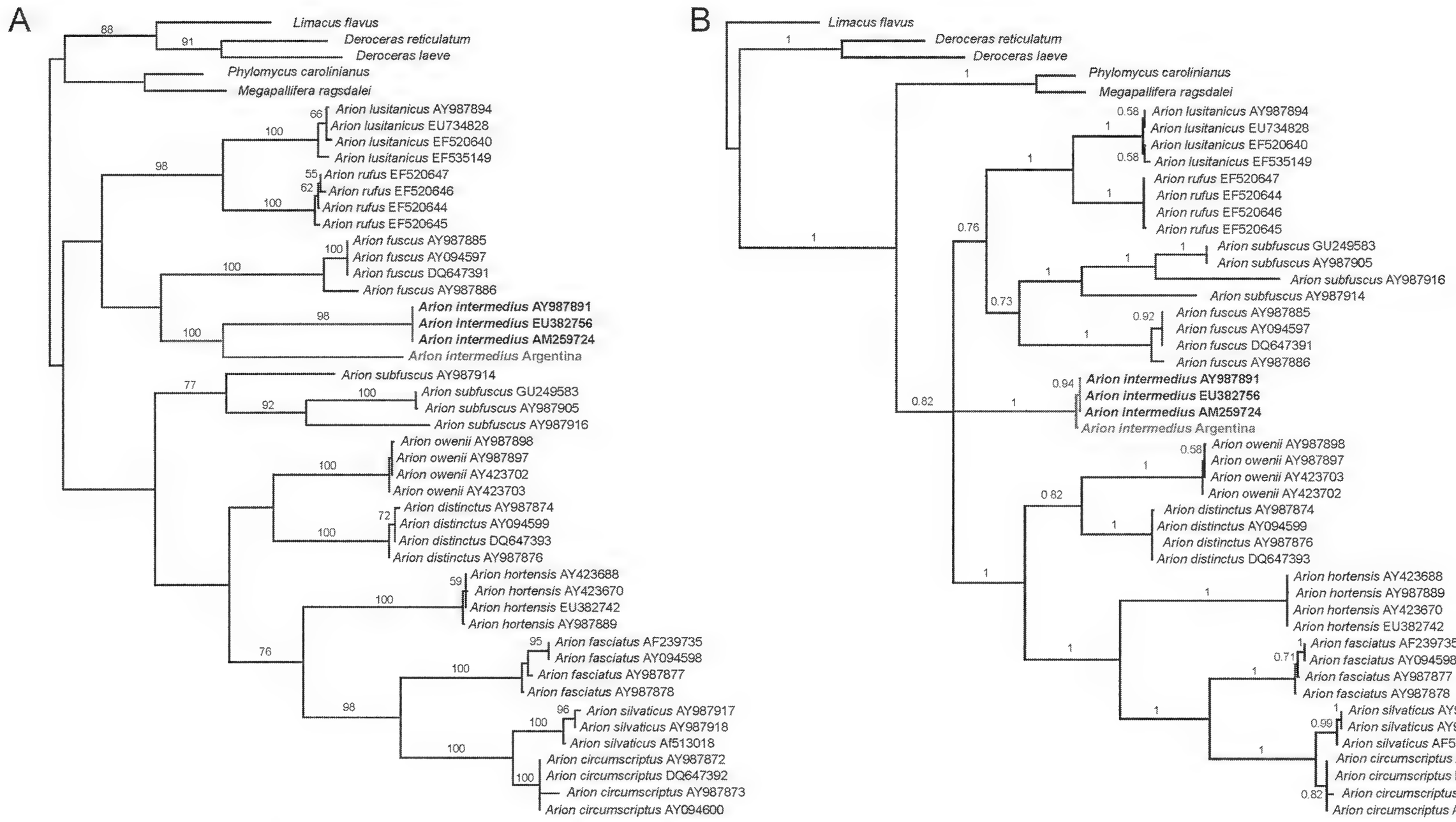

Figure 3. Phylogenetic trees of several invasive or potentially invasive *Arion* species in South America based on 552 nucleotides of the partial COI gene. **A,** One of the two most parsimonious trees (229 parsimony-informative characters, total length = 1074, CI = 0.4181, RI = 0.8085 and, RC = 0.3380). **B,** Bayesian consensus tree. The support values: bootstrap values (MP) and posterior probabilities (BI) are shown on the branches. References to the sequences are given in Table 3.

in agreement with Tsai *et al.* (2011; Fig. 4, cf. the MP and BI trees). In all instances, three groups were identified for *M. pictum*: Group 1 (NJ = 81 , MP = 86, ML = 88, BI = 0.55) was geographically restricted to Taiwan Island (TWP1, TWP2, TWP3, TWP5, TWP6, TWP7, TWP8, TWP9 and TWP 10); Group 2 (NJ = 100, MP = 100, ML = 100, BI = 1) comprised *M. pictum* from Kinmen (TWP11) and Lieyu (TWP12) Islands as well as the South-American specimens (BRP1, BRP2, BRP3, BRP4, BRP5, ARP1 and ARP2) for which only a single haplotype was found. One sequence from southern Taiwan Island (TWP4) and a sequence from continental China (CNP2) were also found within Group 2. Finally, Group 3 (NJ = 99, MP = 91, ML = 100, BI = 0.81) included sequences from continental China (CNP1), Thailand (TLP1) and Matsu Island (*i.e.*, TWP13, TWP14, TWP15).

DISCUSSION

The present study revealed the existence of established populations of four exotic slugs in Argentina. The taxonomic identification of the alien species reported here is based on anatomical studies in addition to sequences of COI gene and constitutes the first report of the presence of these species, all four of which have already shown an invasive behavior in several South American countries. *Deroceras panormitanum* (Lessona and Pollonera, 1882) and *Lehmannia valentiana*—both native of Europe—had been previously recorded for Chile and Colombia, while *L. valentiana* had also been reported in Brazil and Peru (Rumi *et al.* 2010). Based on anatomical and molecular evidence Reise *et al.* (2011) had split *D. panormitanum* into several species, including *D. panormitanum s.s.,* as well as *Deroceras invadens*, that latter as the single successful invader worldwide. According to this new taxonomic arrangement, the classification of *D. panormitanum* remains restricted to only the area where was originally described (*i.e.*, Malta and Sicily). *Arion intermedius*, a typical European Arionidae, has already been recorded in Chile and Colombia (Hausdorf 2002, Letellier *et al.* 2003, Cádiz and Gallardo 2007). *Meghimatium pictum*, a native from eastern and southern Asia, had recently been reported as an alien species in southern Brazil, with that record being the first one of the genus in South America (Gomes *et al.* 2011).

Lehmannia valentiana is the species with the oldest occurrence records in Argentina in comparison to the other three reported here, with data as early as 1924 (Buenos Aires

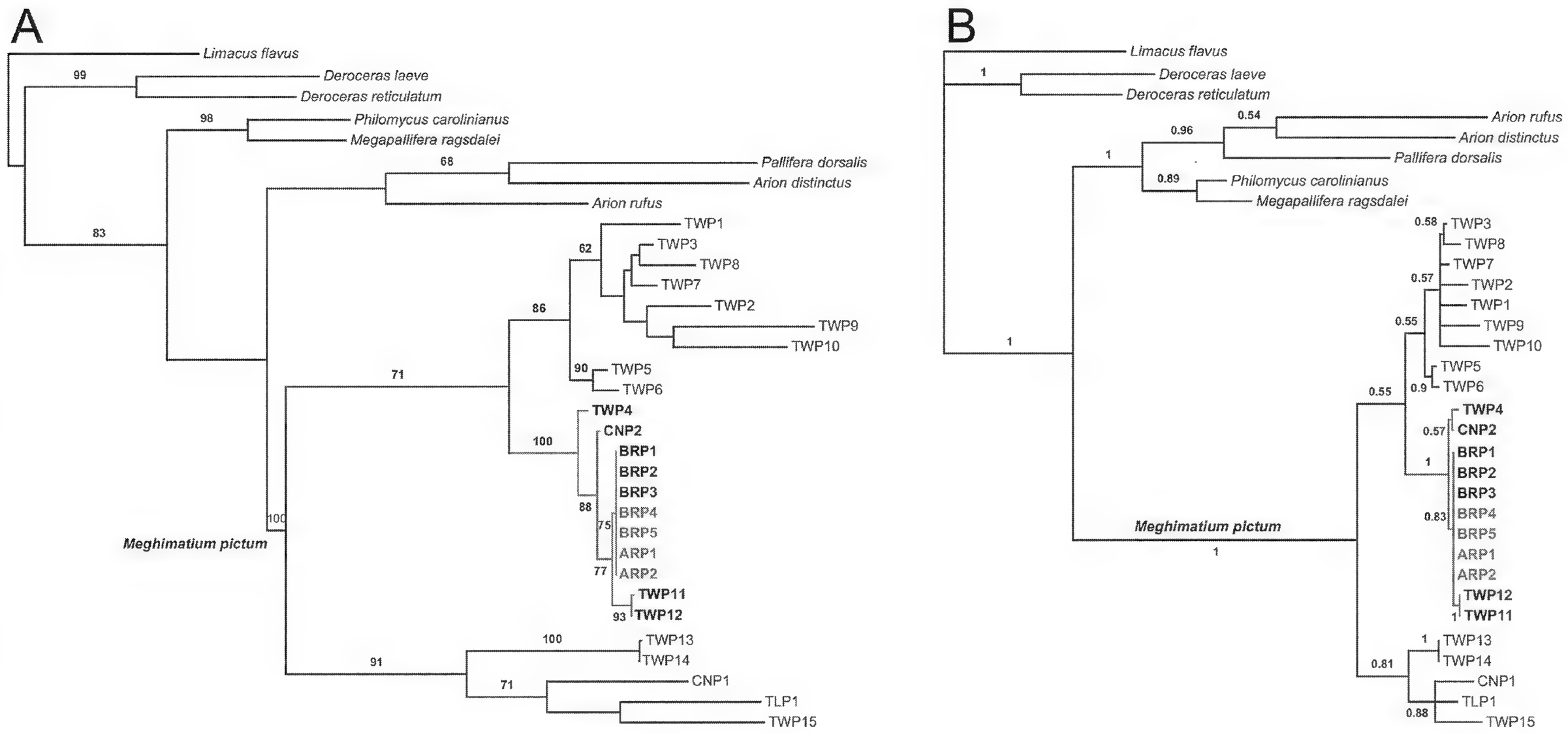

Figure 4. Phylogenetic reconstruction of *Meghimatium pictum,* based on 654 nucleotides of the partial COI gene. **A,** One of the six most parsimonious trees (245 parsimony-informative characters, total length = 987, CI = 0.464, RI = 0.6399 and, RC = 0.2969). **B,** Bayesian consensus tree. The support values: bootstrap values (MP) and posterior probabilities (BI) are shown on the branches. References to the sequences are given in Table 4.

province, MACN-In 14602) and 1962 (Tucumán province, IFML 604). To determine when and how this species was introduced into the country, however, is difficult because the molecular information at hand was not enough to shed any light on the area of origin of the species. *Lehmannia valentiana* could be considered as an established species, with an incipient invasive behavior since specimens have been recorded in remote sites (in both urban and protected areas), feeding on ornamental plants. In other countries, this species is considered to be a greenhouse pest, damaging orchid flowers and ornamental plants (Chichester and Getz 1969, South 1992, Ester *et al.* 2003).

Deroceras invadens was recorded in southern Argentina; but inferences about the site of the species' introduction were not possible because the sequence from Argentina grouped into a clade that included samples from diverse geographical origins (*e.g.,* Canada, U.S.A., Germany, South Africa), thus indicating that the species had already been widely distributed worldwide. The specimens examined were from several towns of southern Argentina, a distribution that suggested the species had acquired an invasive behavior. The probable pathway for the introduction of *D. invadens* into Argentina could be related to commerce, as has been reported in the United States (Meissner *et al.* 2009). *Deroceras invadens* feeds on a great variety of plants and organic material in decomposition and is known to be a pest in pastures, gardens and agricultural fields (Barker 1999). Moreover, this slug species is identified as an intermediate host for the nematode *Gallegostrongylus australis* Spratt, Haycock and Walter, 2001 (Spratt *et al.* 2001), which species parasitizes rodent lungs, causing mild pathologic changes.

We could neither clarify the source location nor assign a precise date of introduction for the specimens of *Arion intermedius* studied. Cádiz and Gallardo (2007) had reported this species for the first time in neighboring Chile, but the date cited could be considered controversial because Letelier *et al.* (2003) had recorded many specimens of *A. intermedius* deposited into National Museum of Natural History of Chile (but with no collection number) from southern Chile. We could nevertheless, conclude that populations of *A. intermedius* had been living in Argentina since at least 2003, the year when they were first collected (Table 1). This species can be considered to be established in Argentina since live specimens had also been found at the same locations seven years later, during the summer 2010 (Table 1). The pathway of introduction of the species into southern Argentina, however, remains unclear. Cádiz and Gallardo (2007) could not establish the introduction pathway into Chile, although they concluded that human accidental transport was the probable cause since species of that genus were being recovered from U.S. seaports on limestone, machinery and rocks (Meissner *et al.* 2009). *Arion intermedius* exhibits polyphagous or phytophagous

feeding habits and a predominant autofecundation, a strategy that increases its invasive capabilities (Wiktor *et al.* 2000, Reise *et al.* 2001). They are known to have a high capacity for penetrating undisturbed native forest, more so than any other type of exotic slug species (Hausdorf 2002, Cadiz and Gallardo 2007). In Argentina, occurrences of the species were recorded within national park areas, far away from urban environments. Within the Arionidae, the genus *Arion* Férussac, 1819 is recognized by international organizations concerned with pest control, such as the Eastern Region Cooperative Agricultural Pest Survey, as potentially damaging for agriculture (Cádiz and Gallardo 2007). Specimens could also act as intermediate hosts for nematodes such as *Filaroides martis* (Werner, 1783), a parasite that affects the respiratory tract of mammals (Grewal *et al.* 2003). In both the most parsimonious tree and the Bayesian consensus tree (Fig. 3), the *Arion* species analyzed grouped in a single clade, suggesting that phylogenetic analysis is as reliable a tool as the morphology in corroborating the identity of the different invasive members of the *Arion* genus.

The introduction of *Meghimatium pictum* into South America, and particularly into Brazil, was suggested to have been accidental through agricultural products in the 1990s, coinciding with the beginning of trade between China and Brazil in order to boost the production of mushrooms in the latter country (Gomes *et al.* 2011). On the basis of position of the South-American samples in all the topologies obtained in our phylogenetic reconstructions, we suggest that the origin of the South-American invasive lineage might be located in a region west of the Strait of Taiwan, either along the coast of China or within the islands close to that coast such as the Taiwanese Kinmen and Lieyu Islands. Currently, *M. pictum* can be found in several states of Brazil (Paraná, Santa Catarina and Río Grande do Sul) bordering northeast Argentina and also in São Paulo State (Gomes *et al.* 2011). Occurrences of this species in the Misiones province of Argentina suggest that the introduction pathway into Argentina could possibly be linked either to an active dispersion of the slug from Brazil and/or to the commerce of flora between those aforementioned Brazilian areas and Argentina. That we recorded one specimen of *M. pictum* in a garden with orchids collected from the Misiones province plus other plants acquired from nurseries in the Tucumán province supports our hypothesis about the commercial trade as being the principal vector of introduction.

In Brazil, *Meghimatium pictum* was associated with the attack of plants in private gardens in Santa Catarina (Gomes *et al.* 2011) and has affected grape vines in Rio Grande do Sul (Baronio *et al.* 2011). In Argentina, further studies are required to know if incipient grape vines in Misiones province, covering about 50 hectares, are already affected; though, the extent of the invasion could be even worse if the species has reached the western provinces (Mendoza, San Juan, La Rioja and Catamarca) where the 97% of the domestic production of wine is concentrated. Such a development could have serious implications on the country's national and international wine trade. In the Argentine areas where *M. pictum* is present, several other gastropods species (both natives and exotics) that can act as intermediate hosts for the zoonotic parasitic nematode *Angiostrongylus costaricensis* Moreira and Céspedes, 1971 can be found, with the potential risk that *M. pictum* could likewise begin to act as a new vector. *Meghimatium bilineatum* (Benson, 1842), a very close species to *M. pictum*, has furthermore been reported to be a host of *Angiostrongylus cantonensis* (Cheng, 1935) (Li *et al.* 2006), another zoonotic parasitic nematode.

We provide here the first report of four new exotic slugs in Argentine territories. Because of the invasive potential of these slug species, the present work provides new COI sequences obtained from morphologically confirmed specimens. We believe that the information contained in this report can help nonspecialists and government authorities quickly identify these species for the purpose of establishing guidelines for the prevention, control, and diffusion of those alien slugs.

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Phylogenetic analysis of the freshwater mussel genus *Ptychobranchus* (Bivalvia: Unionidae)

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Abstract: The phylogenetic relationships of species of the mussel genus *Ptychobranchus* Simpson, 1900 were examined using the mitochondrial DNA sequences of the ND1 and 16S gene regions. A total of 31 individuals representing the five species are included in this analysis. Outgroups were drawn from other unionid genera previously shown to be closely related to *Ptychobranchus*. Phylogenetic analysis was conducted using Bayesian methods applying several data-partitioning strategies. The results of all analyses support the monophyly of *Ptychobranchus*, and the interrelationships of its constituent species are consistent across all analyses. *Ptychobranchus occidentalis* (Conrad, 1836) is recovered as paraphyletic with *P. fasciolaris* (Rafinesque, 1820). Molecular analyses indicate that *Ptychobranchus jonesi* van der Schalie, 1934 is a member of the genus, and character state reconstruction predicts that it should possess the complex conglutinate that is found in other species of *Ptychobranchus*.

Key words: Systematics, Bayesian, Mollusca, Unionoida, DNA

The North American unionid genus *Ptychobranchus* Simpson, 1900, commonly referred to as kidneyshells, is generally considered to consist of five species: *P. fasciolaris* (Rafinesque, 1820), *P. greenii* (Conrad, 1834), *P. jonesi* (van der Schalie, 1934), *P. occidentalis* (Conrad, 1836), and *P. subtentum* (Say, 1825) (Fig. 1) that are distributed across the interior of North America (Fig. 2). Conchologically variable in size, shape and color, the members of this genus were originally united taxonomically because they possess the same distinctive demibranch morphology when gravid (Simpson 1900a, Ortmann 1910, Fuller and Bereza 1973) (Fig. 3A–D). In all unionoid bivalves, females brood their larvae in modified portions of one or both pairs of demibranchs. Within the unionid tribe Lampsilini, of which *Ptychobranchus* is a member, water tubes of the demibranchs expand to varying degrees as the glochidia mature. The shape and degree of the expanding water tubes are distinctive to sub-groups within the tribe (Ortmann 1912). In *Ptychobranchus*, the ventral portion of the water tubes in the outer demibranchs are expanded to a greater degree than the dorsal edges, creating a series of undulations so that in a fully gravid female the entire structure of each demibranch resembles a folded curtain.

Many genera of North American unionids have been defined based on characters related to reproduction (Ortmann 1910, 1912). Reproduction in unionoid bivalves is remarkable in that the glochidia must attach to a vertebrate host in order to complete metamorphosis to the juvenile stage (Lefevre and Curtis 1912), and many species have evolved lures that are used to attract potential hosts (Barnhart *et al.* 2008). The development of such lures attains its zenith in the members of the unionid tribe Lampsilini, wherein a number of species package their larvae into the lure itself, such structures are referred to as conglutinates, and it can be argued that among lampsiline mussels the genus *Ptychobranchus* has evolved the most elaborate kind of conglutinates. In *Ptychobranchus*, conglutinates consist of several acellular layers that surround a central core of glochidia (Watters 1999). The entire structure is pigmented and possesses an adhesive distal end that when attached to a rock or other suitable hard surface enhances the hypothesized mimicry of a larval fish or insect. Images and video of the lures of *P. occidentalis* can be viewed at the URL: http://unionid.missouristate.edu/gallery/ouachita/kidneyshell.htm. Once a fish attempts to ingest the conglutinate, pressure from its jaws ruptures the outer layer and causes the glochidia to be expelled into the pharyngeal cavity of the potential host (Barnhart and Roberts 1997) where they may subsequently attach to the gills of the fish. The conglutinates of *P. fasciolaris* are virtually identical to those of *P. occidentalis* in size, shape and coloration, and are thought to mimic fish larvae (Barnhart and Roberts 1997). Conglutinates produced by *P. greenii* resemble either dipteran larvae (Hartfield and Hartfield 1996) or fish eggs (Haag and Warren 1997), whereas the striking conglutinates produced by *P. subtentum* resemble the pupae of members of the dipteran family Simulidae (http://unionid.missouristate.edu/gallery/Psubtentum/fluted.htm). There are no published accounts or descriptions of the conglutinates produced by *P. jonesi*.

Simpson's (1900a) original description of the genus *Ptychobranchus* did not include two currently recognized species, *P. subtentum* and *P. jonesi*. *Ptychobranchus subtentum*

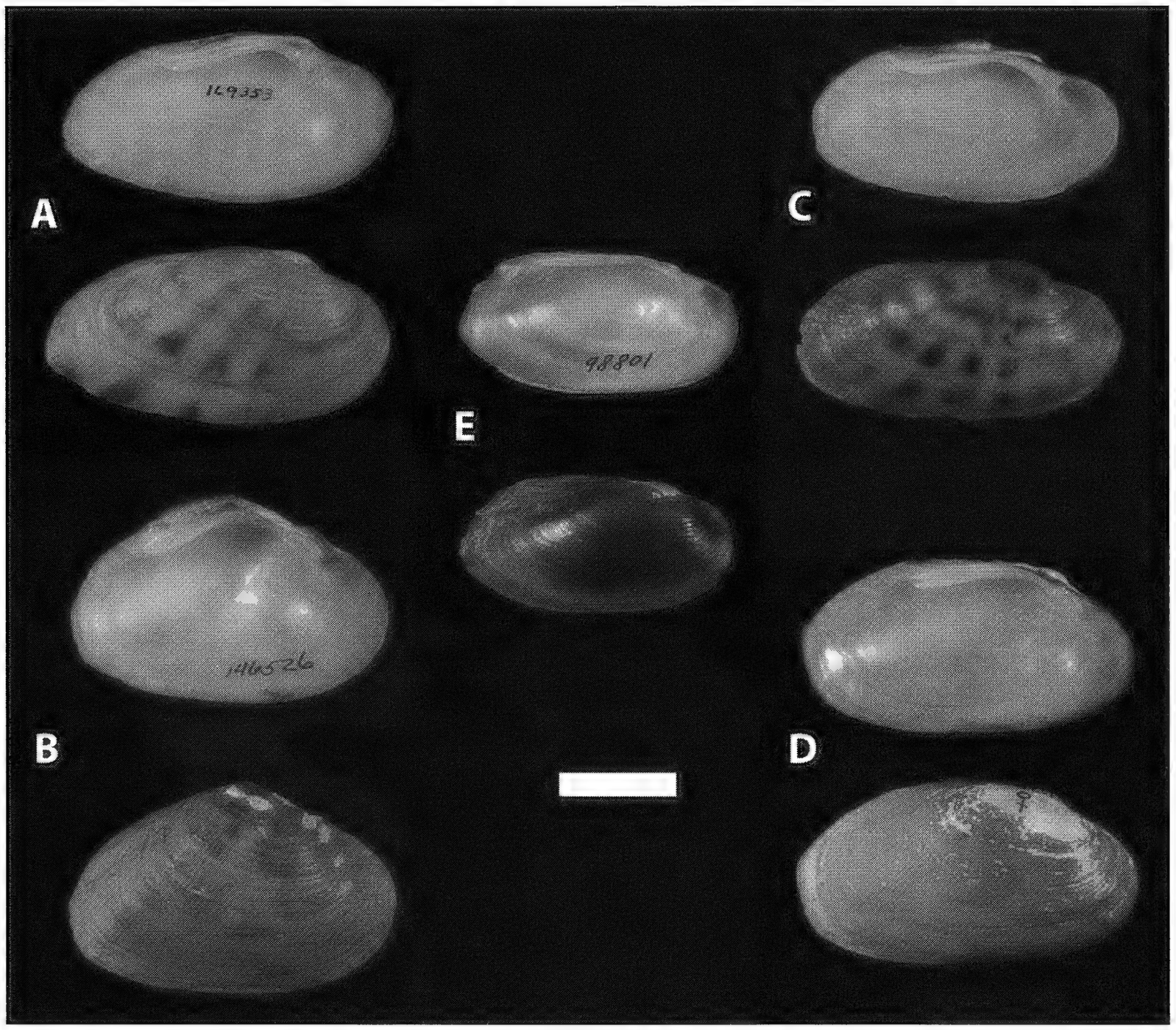


Figure 1. Sample of and internal and external conchological variation in *Ptychobranchus*. **A,** *P. fasciolaris*; **B,** *P. greenii*; **C,** *P. subtentum*; **D,** *P. occidentalis*; **E,** *P. jonesi*. Scale bar = 20 mm. All specimens from the Delaware Museum of Natural History.

had been previously placed in the genus *Medionidus* Simpson, 1900 by Simpson (1900a) because the shells of this species possess a "corrugated" posterior slope, which is characteristic of that genus, and only conchological material was available for examination at the time of his description (Simpson 1900b). *Ptychobranchus subtentum* was later moved to *Ptychobranchus* by Ortmann (1912). *Ptychobranchus jonesi* was originally placed in the genus *Lampsilis* Rafinesque, 1820 by van der Schalie (1934) and was later incorrectly synonymized with another lampsiline, *Hamiota australis* (Simpson, 1900) (Simpson 1900a) by Clench and Turner (1956). The incorrect synonymy with *H. australis* was later rectified by Athearn (1964), but it would be a decade before Fuller and Bereza (1973) would briefly note in a meeting abstract that specimens of *P. jonesi* possess the folded outer demibranchs typical of the genus *Ptychobranchus*.

No formal phylogenetic analysis of the genus *Ptychobranchus* has been conducted to date, although several studies on unionoids at higher taxonomic levels have included up to two members of the genus (Campbell *et al.* 2005, Zanatta and Murphy 2006, Bogan and Roe 2008). These studies indicate a phylogenetic affinity of *Ptychobranchus* with the genera *Cyprogenia* Agassiz, 1852, *Dromus* Simpson, 1900, and *Medionidus*. The purpose of this study is to explicitly test the monophyly of *Ptychobranchus* and the synapomorphic status of the complex conglutinates. In addition, this study will result in hypotheses of relationships for the species within the genus, *Ptychobranchus*, using molecular data.

MATERIALS AND METHODS

DNA sequences

Tissue samples were collected from 29 new specimens representing the five widely recognized species of *Ptychobranchus*. Locality information, museum catalog numbers and GenBank accession numbers are provided in Appendix 1. Genomic DNA was extracted from mantle tissue from frozen or ethanol-preserved specimens using standard proteinase K/SDS digest (Roe and Lydeard 1998) or the DNeasy blood and tissue kit (Qiagen). A ~700 base-pair (bp) region of the 5′-end of the first subunit of the mitochondrial NADH dehydrogenase (ND1) gene was amplified using primers Leu-uurF and NIJ-12073 (Serb *et al.* 2003), and ~500 bp region of the mitochondrial 16SrDNA gene was amplified using the primers from Lydeard *et al.* (1996). Thermal cycling for double-stranded amplification used the following conditions: 34 cycles of denaturing (95 °C, 40 s), annealing (50–58 °C, 1 min), and elongation (72 °C, 1.5 min). PCR products were purified using spin filtration columns (Millipore ultra-free-mc No.UFC3 LTK00). Purified PCR products were used as template for cycle sequencing reactions with the ABI Prism Big Dye Terminator kit (v. 2.0, Applied Biosystems). Cycle sequencing reactions were cleaned by DyeEx Spin kit (Qiagen), resuspended in 10 µL of formamide, and read by an ABI 3100 automated sequencer. Sequences were initially entered into the software program BioEdit (Hall 1999) and visually edited and aligned. Alignment of the 16SrDNA sequences was accomplished using ClustalW (Thompson *et al.* 1994) using default settings and the alignment was later modified by eye. The ND1 portion of the dataset was aligned by eye and converted into amino acid sequence to check the accuracy of the nucleotide sequence alignment. Sequences generated for this study were combined with available sequence data for one specimen of *P. subtentum* and one

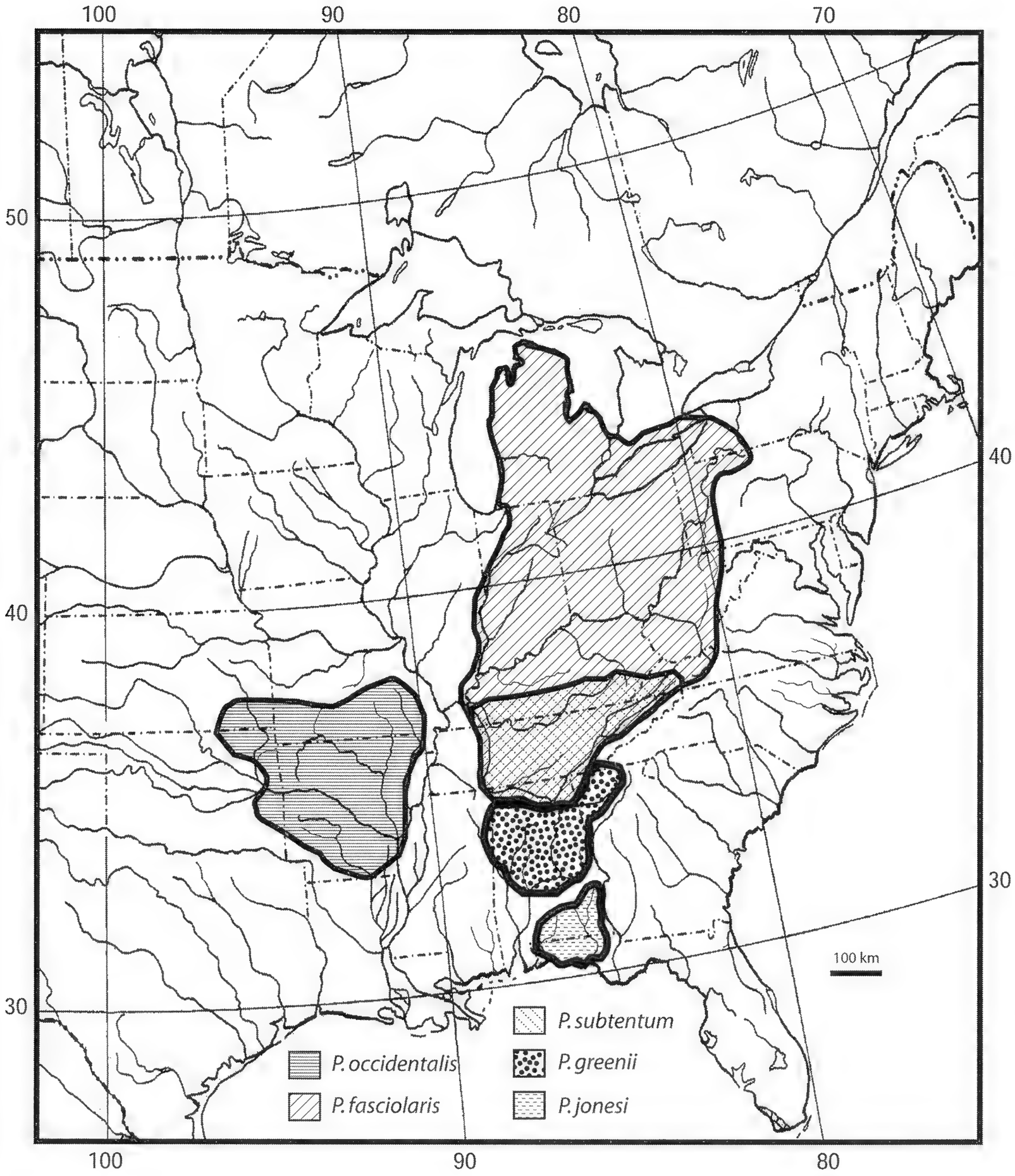


Figure 2. Map of North America showing the geographic distribution of the five species of *Ptychobranchus*.

specimen of *P. fasciolaris* from GenBank for a total of 31 sequences. An additional 12 sequences from GenBank representing the six outgroup taxa were also included in the data set for a total of 37 sequences. GenBank accession numbers for all specimens are listed in Appendix 1.

Morphology

A matrix consisting of 19 characters representing conchological, anatomical, and larval features was constructed for species of *Ptychobranchus* and outgroup taxa. Fourteen of these characters were derived from Graf and Cummings (2006). Character states were scored for all taxa by the author through the examination of multiple museum specimens. Because the majority of the characters examined were either invariant within *Ptychobranchus* or polymorphic within a species, the morphological matrix was not included in any phylogenetic analyses. However, character state reconstruction via Mesquite 2.75 (Maddison and Maddison 2011) was used to predict the most parsimonious reconstruction of the conglutinate type for *P. jonesi*.

Bayesian analysis

A starting tree was created for the entire data set using the Maximum Likelihood (ML) optimality criterion and the Jukes Cantor (JC) model (tree-bisection and reconnection (TBR) branch swapping and 10 random addition replicates) in PAUP* (Swofford 2002). The data set was partitioned based on gene identity (16S or ND1) and structural or functional constraints (stems vs. loops and codon position). RNA secondary structure predictions were performed using the web application GeneBee (http://www.genebee.msu.su/services/rna2_reduced.html) to identify stem and loop regions of the gene for partitioning during analyses (Table 1). The appropriate model of sequence evolution for each partition and combination of partitions was determined using the JC tree and the likelihood ratio test (LRT) as implemented in MrModeltest 2.3 (http://www.abc.se/~nylander/).

All phylogenetic analyses were conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Analyses were conducted for each partitioning strategy (Table 2) starting with a random tree and default priors. Four Markov chains were sampled every 1000 generations and each analysis consisted of between 2.0 x 10^7 to 4.0 x 10^7 generations (Table 2). Analyses were monitored by evaluating the average standard deviation of split frequencies reported by MrBayes, and the cumulative posterior probabilities using the *cumulative* and *compare* options in the web-based application AWTY (Nylander *et al.* 2008). Analyses were discontinued after stationarity was detected. Stationarity was assumed when standard deviation of split frequencies dropped below 0.001 and the cumulative posterior probabilities stabilized. Trees generated prior to stabilization (burnin) were discarded. Evaluation of the different partitioning strategies was accomplished by comparing the mean $-\ln L$ and the Bayes factor (BF) for each partitioning strategy (Kass and Raftery 1995). Bayes factors (BF) were calculated using

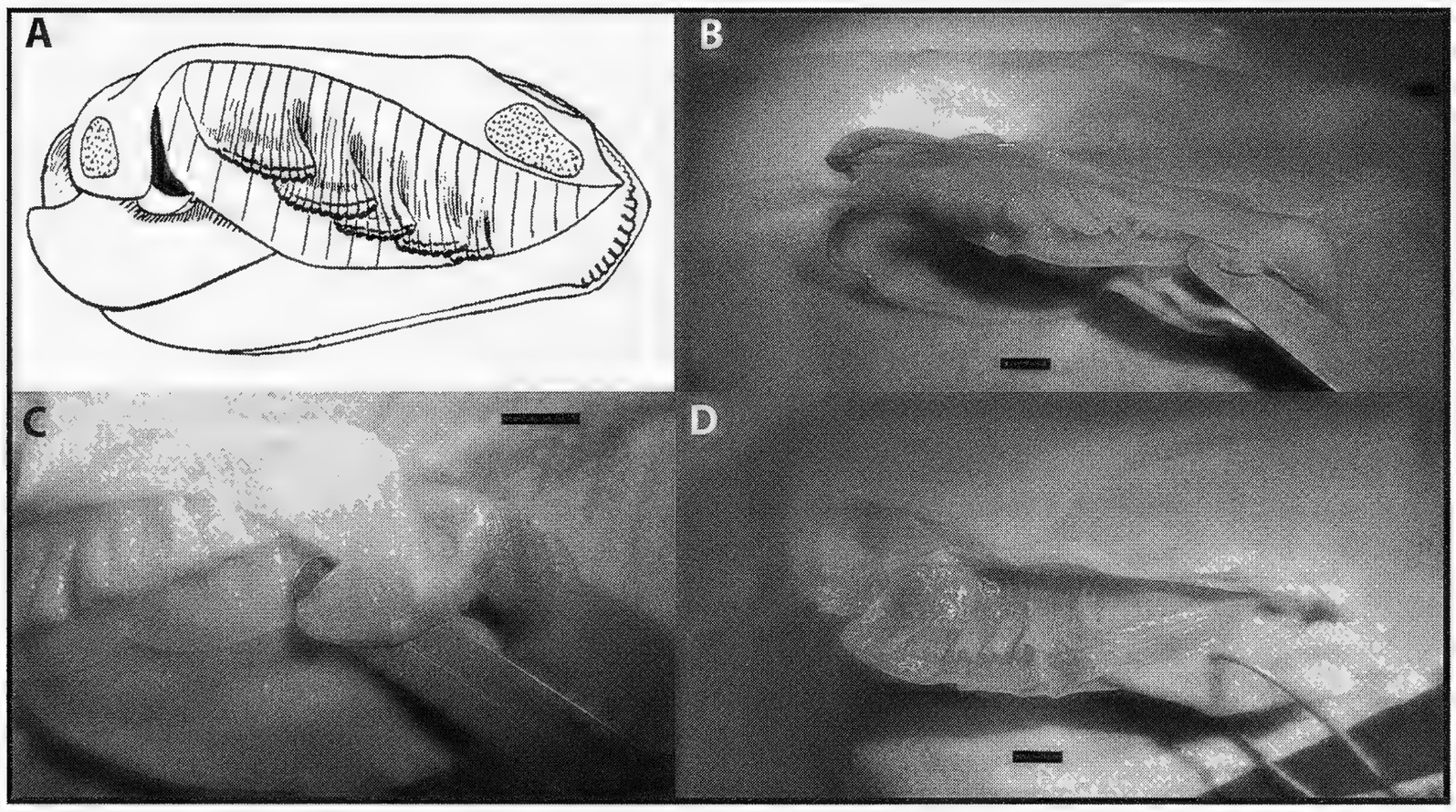


Figure 3. Illustration and examples of the outer demibranchs of species of *Ptychobranchus*. **A,** Illustration of fully gravid *P. subtentum* from Ortmann 1912; **B,** Fully gravid *P. subtentum* (INHS 13531); **C,** *P. fasciolaris* at early stage of gravidity (No Cat. #); **D,** *P. jonesi* (INHS 11886). Scale bar = 5 mm.

harmonic means (hm) of the likelihood values for each analyzed partition strategy that were generated using the *sump* command in MrBayes and the formula for calculating BF in Brandley *et al.* (2005). Consensus topologies of post-stationarity trees were created in MrBayes and prepared for publication using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

The 16S portion of the data set consisted of 442 aligned nucleotides and the ND1 portion consisted of 645 nucleotides for a total of 1087 base pairs, of which 351 were variable and 243 were phylogenetically informative. Models selected and the numbers of characters in each partition are presented in Table 1. A comparison of the mean –ln*L* from the analysis of each partition strategy reveals that partitioning the 16S portion of the data set into stem and loop regions did not result in an improvement of the mean –ln*L* score, whereas partitioning the ND1 portion of the data set by codon region does improve the mean –ln*L* (Table 3). Although partitioning the data set by codon position seemed to result in an improved mean –ln*L*, the different partitioning strategies did not result in appreciably different consensus topologies (Fig. 4). All four of the consensus topologies were very similar, and the observed differences were restricted to "tip" regions of the topologies. As might be expected due to the similarity in consensus topologies between analyses, comparing the results using the BF reveals no single phylogenetic hypothesis was strongly supported over any other (Table 4); BF values > 6 would indicate that the alternative hypothesis was strongly favored (Kass and Raferty 1995). All analyses resulted in a monophyletic *Ptychobranchus* with weak to moderately strong posterior probabilities (PP) depending on partition strategy. Interestingly, PP decreased for the node supporting the monophyly of *Ptychobranchus* when the ND1 portion of the data set was partitioned by codons. All species of *Ptychobranchus* with the exception of *P. occidentalis* were strongly supported as monophyletic. All analyses placed *P. subtentum* as the most basal member of the genus (PP = 0.54–0.76), sister to the remaining four species. *Ptychobranchus jonesi* was the next most basal clade (PP = 0.37–0.44), followed by *P. greenii* (PP = 0.98–0.99), which was sister to a paraphyletic *P. occidentalis*, plus *P. fasciolaris*. The *P. occidentalis* + *P. faciolaris* clade is strongly supported (PP = 1.0 in all analyses), but relationships between the three well-supported clades of *P. fasciolaris* and *P. occidentalis* from the Saline River (Ouachita Highlands) and the remaining *P. occidentalis* from the Ozark Highland drainages are supported by PP ≤ 0.59.

The consensus trees resulting from Bayesian analyses were trimmed so that each species (or clade in the case of *Ptychobranchus occidentalis*) was represented by a single terminal. The resulting topology was used to reconstruct the evolution of conglutinate type, which was treated as a single, unordered binary character for reconstruction of states, *i.e.* complex conglutinate present or absent. The most parsimonious reconstruction (not shown) predicts that the complex conglutinate evolved in the ancestor of *Ptychobranchus* and that all species of *Ptychobranchus* including *P. jonesi* possess complex conglutinates.

Table 1. Data partitions, selected models of sequence evolution, and partition size.

Partition	Model	Number of characters in partition
All data	GTR+I+G	1087
16S	GTR+I+G	442
16S stems	SVM+G	142
16S loops	GTR+I+G	300
ND1	GTR+I+G	645
ND1 1st codon	GTR+G	215
ND1 2nd codon	GTR+I	215
ND1 3rd codon	HKY+I+G	215

Table 2. Partitioning strategies used in this study, and duration of analysis in generations.

Partition strategy	Partition identity	Number of generations to achieve stationarity
P1	All data combined	3.0×10^7
P2	16S stems, 16S loops, and ND1	2.0×10^7
P3	16S, ND1 1st, ND1 2nd, and ND1 3rd	4.0×10^7
P4	16S stems, 16S loops, and ND1 1st, ND1 2nd, and ND1 3rd	4.0×10^7

DISCUSSION

The molecular phylogenetic hypotheses presented in this study all indicate that *Ptychobranchus* as currently conceived, is a monophyletic group. These results also indicate that the unique demibranch morphology is a synapomorphy for the genus. The reconstructions of ancestral characters states predict that the complex conglutinates are also a synapomorphy for the genus. In addition, all analyses strongly support all but one species as monophyletic. *Ptychobranchus occidentalis* was recovered as paraphyletic with respect to *P. fasciolaris*. The identification of distinct Ozark and Ouachita Highland lineages of aquatic organisms is not without precedent (*e.g.*, Mayden 1988, Crandall and Templeton 1999). Whether the two clades of *P. occidentalis* identified here warrant recognition as distinct entities will need to be confirmed by more extensive geographic sampling and larger sample sizes.

It has been recommended by Williams *et al.* (2008) that the *Ptychobranchus greenii* of the Mobile Basin be recognized as consisting of two distinct entities: *P. formanianus* (Lea, 1842), and *P. greenii*, inhabiting the Coosa and Tallapoosa rivers and the Tombigbee and Black Warrior rivers, respectively. The distinction by Williams *et al.* (2008) was made based on the presence of well-defined dark green rays on the periostracum of *P. formanianus*, which are lacking in *P. greenii*. While the present study does resolve individuals from the ranges of these two putative species as reciprocally monophyletic, it should be noted that the sample sizes are small, and additional work will be needed for a formal test of species status for these two clades.

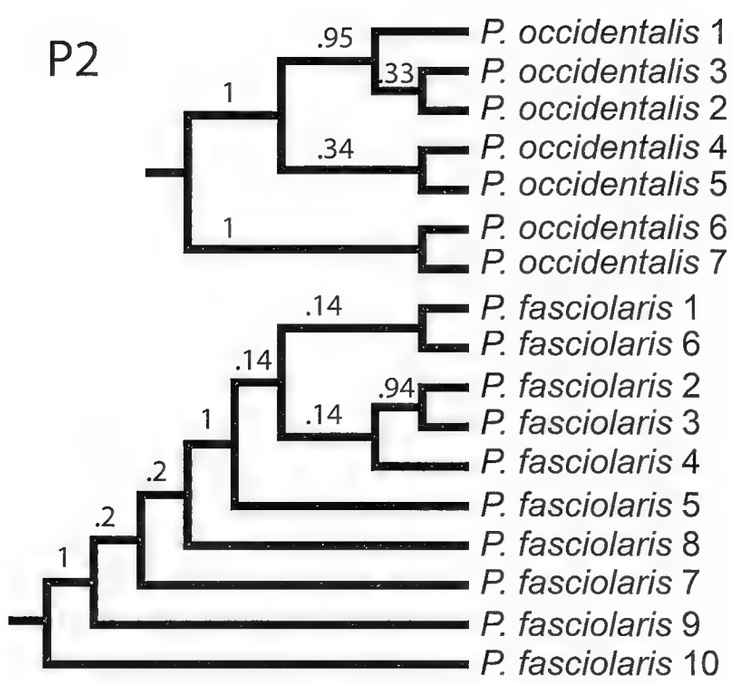
Prior to this study, there was little information available that indicated that *P. jonesi* was a member of the genus *Ptychobranchus*, and to date, no conglutinates have been described for *P. jonesi*. *Ptychobranchus jonesi* is an extremely rare species (Gangloff and Hartfield 2009) that is not widely represented in natural history collections, and preserved examples that would allow examination of the demibranchs of this species are rare. The author did locate and examine a preserved specimen of *P. jonesi* (INHS 11886) that, based on the degree of demibranch folding and swelling, appears to manifest the outer demibranch morphology of a female in the early stages of gravidity (Fig. 3D). Furthermore, all phylogenetic hypotheses generated in this study place *P. jonesi* as the second most basal clade in the genus, sister to a group consisting of all other species with the exception of *P. subtentum*. Character state reconstruction of conglutinate type using the phylogeny generated in this study predicts unambiguously that *P. jonesi* would also possess a complex conglutinate typical of *Ptychobranchus*.

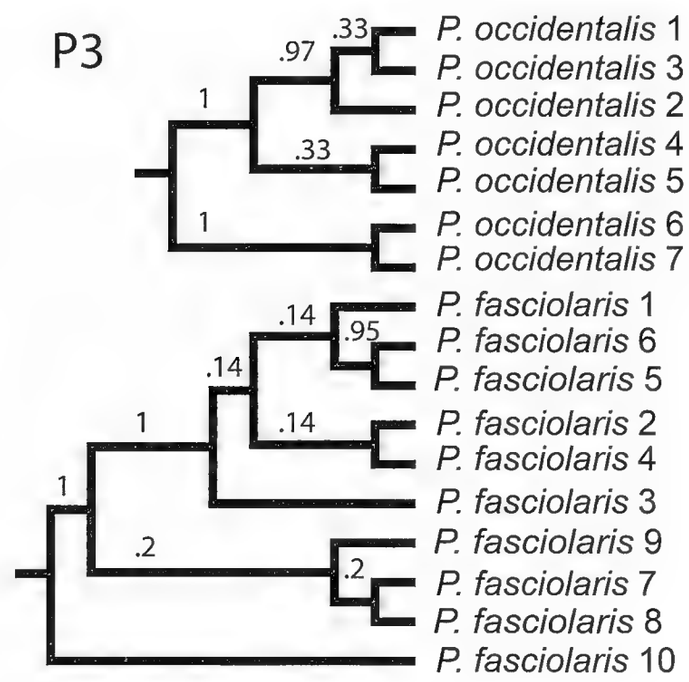
Ptychobranchus subtentum is recovered as the most basal member of the genus *Ptychobranchus*. Some early taxonomists (Simpson 1900b) had placed *P. subtentum* in the genus *Medionidus*, because most individuals possess a corrugated posterior slope. The observation of the typical folded outer demibranchs in specimens by Ortmann (1912) implied that *P. subtentum* had affinities with species placed in *Ptychobranchus*, and is supported by the results of this study.

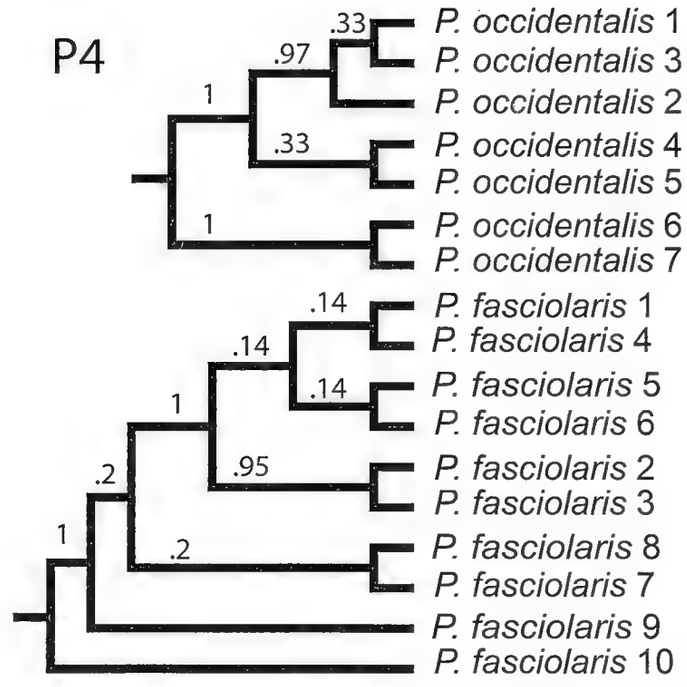
The freshwater mussel family Unionidae is one of the largest families in the Bivalvia and includes over 670 species (Graf and Cummings 2007). Although multiple phylogenetic studies have demonstrated that six major lineages (families) of freshwater mussels comprise the Unionoida (*e.g.*, Hoeh *et al.* 2002, Roe and Hoeh 2003, Graf and Cummings 2006, Walker *et al.* 2006), robustly supported phylogenies of the relationships among these groups are still lacking. This largely stems from a reliance on a small number of molecular markers primarily consisting of the nuclear 28S rDNA gene and 3 mitochondrial gene regions (Bogan and Roe 2008). While these three mitochondrial markers often provide poor support for higher taxonomic groupings within the Unionoida, their performance has generally been better at lower taxonomic levels in the new world Unionidae (*e.g.*, Davis and Fuller 1981, Lydeard *et al.* 1996, Roe and Lydeard 1998, Bogan and Hoeh 2000, Graf and Ó Foighil 2000, Graf 2002, Serb *et al.* 2003, Campbell *et al.* 2005, Walker *et al.* 2006, Zanatta and Murphy 2006, Bogan and Roe 2008). Several of these studies have indicated support for some higher taxonomic groupings (*e.g.*, tribes), however

Table 3. Mean $-\ln L$ and 95% credible intervals for each partitioning strategy.

Partition strategy	Mean $-\ln L$	Upper 95% CI	Lower 95% CI
P1	4852.456	4852.295	4852.479
P2	4854.790	4854.698	4854.883
P3	4722.889	4722.816	4722.961
P4	4742.415	4742.347	4742.484







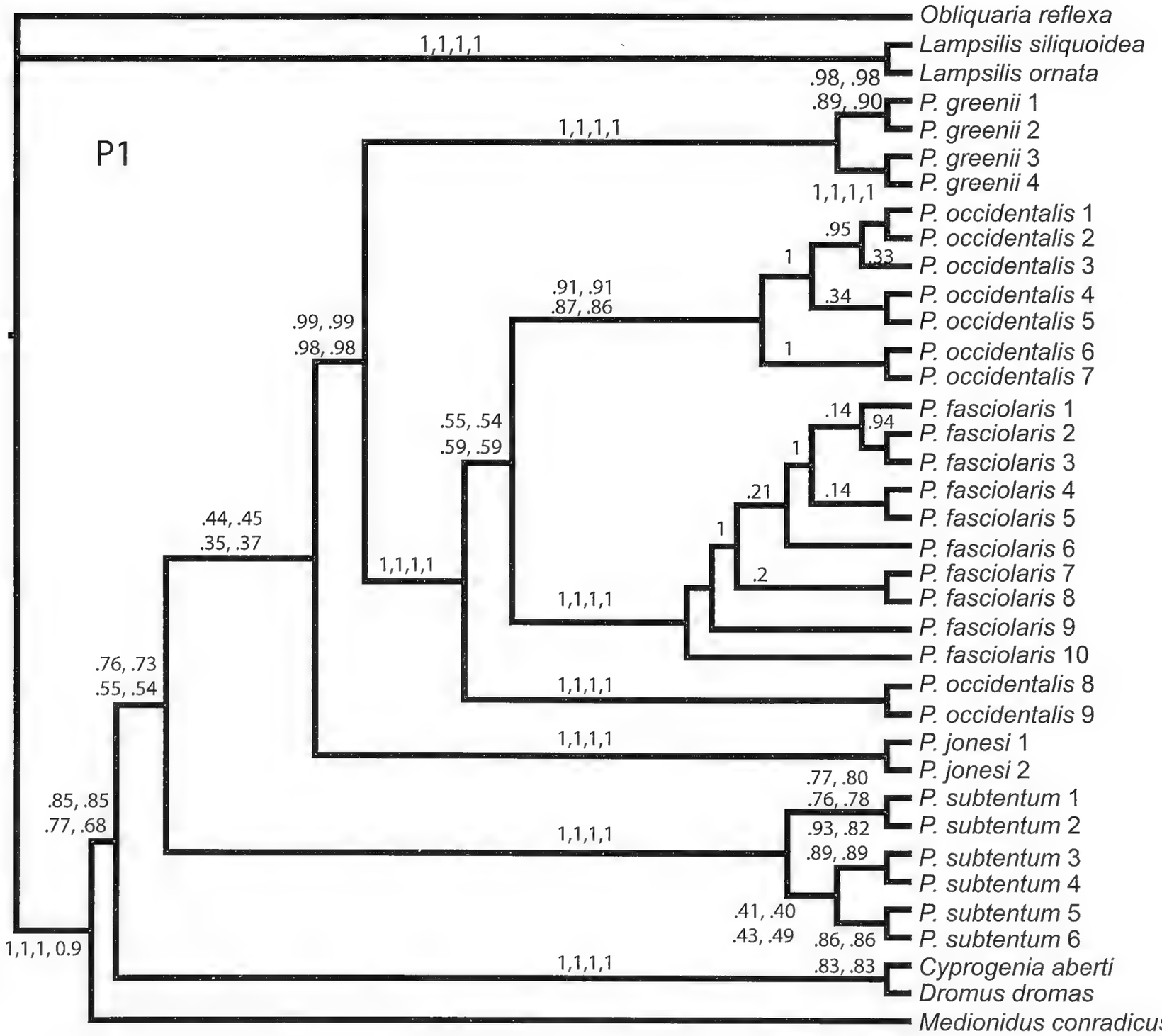


Figure 4. Consensus trees resulting from the partitioned analysis of the data. Labels at nodes are posterior probabilities resulting from the analysis of the partition strategies P1–P4. See Table 2 for details of partition information. Subtrees P2–P4 indicate where these consensus trees resulting from the analysis of those partitions differed from P1.

many of the unionid genera as currently defined, appear to be polyphyletic (*e.g.*, Campbell *et al.* 2005). Genera that to date have been supported as natural (monophyletic) taxa in molecular phylogenetic analyses tend to be those that include relatively few species or possess some distinctive morphological trait, such as the superconglutinate lure shared by members of the genus *Hamiota* Roe and Hartfield, 2005 (Roe *et al.* 2001). This study represents a case in which a genus with a distinctive morphological trait (complex conglutinates) whose monophyly is not supported by high PP. Examination of the four partitioned analyses reveals a trend at several nodes for a decrease in PP as the model complexity increases from P1 to P4. Huelsenbeck and Rannala (2004) examined the effect of under- or over-parameterizing models in Bayesian analyses and found that PP were over-estimated when the model used to analyze the data is underspecified

Table 4. 2ln Bayes factor results of comparisons of all partitioning strategies.

Partition strategy

	P1	P2	P3	P4
P1	-	0.0	0.05	0.44
P2		-	0.05	0.44
P3			-	0.006
P4				-

relative to the model that generated the data. They also found that the when the model was overspecified relative to the model that generated the data, the PP were generally accurate or only slightly overestimated. Based on the findings of Huelsenbeck and Rannala (2004), even if the models in partitions P3 and P4 are overspecified the lower PP values should be considered accurate estimates of the probability that the tree is correct given that the model is correct.

Although the analyses of the molecular data set unambiguously support the monophyly of *Ptychobranchus* regardless of partition strategy, the number of weakly supported nodes in this and other phylogenetic studies of unionoids reinforces the need for the development of additional markers called for by Bogan and Roe (2008). Until such time, the lack of well-supported phylogenies will continue to hinder an improved understanding of the evolutionary relationships of these organisms.

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Appendix 1. Specimens Examined

Species (sample number)	Locality, accession number	River drainage	GenBank no. 16S	ND1
Ptychobranchus fasciolaris (Rafinesque, 1820)				
(1)	Green River, Hart Co., Kentucky, UAUC 2583	Ohio	JX311498	JX311532
(2)	Little Darby Creek, Madison Co., Ohio	Scioto	JX311503	JX311537
(3)	Little Darby Creek, Madison Co., Ohio	Scioto	JX311504	JX311538
(4)	Barren River, Warren Co., Kentucky, INHS 20163-4	Ohio	JX311505	JX311539
(5)	Powell River, Hancock Co., Tennessee	Tennessee	JX311506	JX311540
(6)	Station Camp Creek, Scott Co., Tennessee	Cumberland	JX311507	JX311541
(7)	Fish Creek, Williams Co., Ohio	Maumee	JX311500	JX311534
(8)	Fish Creek, Williams Co., Ohio	Maumee	JX311501	JX311535
(9)	Clinch River, Hancock Co., Tennessee, UAUC 1515	Tennessee	JX311499	JX311533
(10)	Elk River, Kanawah Co., West Virginia, LSC23701001	Kanawah	JX311502	AY655120
Ptychobranchus greenii (Conrad, 1834)				
(1)	Conasauga River, Murray/Whitfield Co., Georgia	Coosa	JX311483	JX311517
(2)	Coosawattee River, Gordon Co., Georgia	Coosa	JX311484	JX311518
(3)	Sipsey Fork, Lawrence Co., Alabama, UAUC 91	Black Warrior	JX311485	JX311519
(4)	Brushy Creek, Winston Co., Alabama, UAUC 546	Black Warrior	JX311486	JX311520
Ptychobranchus jonesi (van der Schalie, 1934)				
(1)	West Fork of the Choctawhatchee River, Barbour Co., Alabama, UAUC 1069	Choctawhatchee	JX311487	JX311521
(2)	West Fork of the Choctawhatchee River, Barbour Co., Alabama, UAUC 1070	Choctawhatchee	JX311488	JX311522
Ptychobranchus occidentalis (Conrad, 1836)				
(1)	St. Francis River, Wayne Co., Missouri, INHS 24216	White	JX311489	JX311523
(2)	Jacks Fork River, Shannon Co., Missouri, INHS 2431	Current	JX311497	JX311531
(3)	St. Francis River, Wayne Co., Missouri, INHS 24216	White	JX311490	JX311524
(4)	Strawberry River, Sharp Co., Arkansas	White	JX311495	JX311529
(5)	Strawberry River, Sharp Co., Arkansas	White	JX311496	JX311530
(6)	Spring River, Cherokee Co., Kansas, LSC23704-001003	Neosho	JX311493	JX311527
(7)	Spring River, Jasper Co., Missouri	Neosho	JX311494	JX311528
(8)	Saline River, Grant Co., Arkansas, INHS 14594-4	Ouachita	JX311491	JX311525
(9)	Saline River, Grant Co., Arkansas, INHS 14594-11	Ouachita	JX311492	JX311526
Ptychobranchus subtentum (Say, 1825)				
(1)	Little South Fork of the Cumberland River, Wayne Co., Kentucky	Cumberland	JX311508	JX311542
(2)	Little South Fork of the Cumberland River, Wayne Co., Kentucky	Cumberland	JX311509	JX311543
(3)	Rock Creek, McCreary Co., Kentucky	Cumberland	JX311510	JX311544
(4)	Rock Creek, McCreary Co., Kentucky	Cumberland	JX311511	JX311545
(5)	Clinch River, Hancock Co., Tennessee, UAUC 11	Tennessee	JX311512	JX311546
(6)	Clinch River, Hancock Co., Tennessee, UAUC 167	Tennessee	JX311513	JX311547
Cyprogenia aberti (Conrad, 1850)	No locality information		JX311516	AY158749
Dromus dromas (Lea, 1834)	Clinch River, Hancock Co., Tennessee, UAUC 1506	Tennessee	AY655033	AY158750
Lampsilis ornata (Conrad, 1835)	Cahaba River, Bibb Co., Alabama, UAUC 17	Alabama	AF385136	AY158748
Lampsilis siliquoidea (Barnes, 1823)	Douglas Lake, Cheboygan Co., Michigan, UAUC882	N/A	JX311515	AY158747
Medionidus conradicus (Lea, 1834)	Clinch River, Hancock Co., Tennessee	Tennessee	JX311514	AF385136
Obliquaria reflexa Rafinesque, 1820	Cahaba River, Bibb Co., Alabama, UAUC 19	Alabama	AF385138	AY158751

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Phylogeography and genetic variability of the freshwater mussels (Bivalvia: Unionidae) Ellipse, *Venustaconcha ellipsiformis* (Conrad 1836), and Bleeding Tooth, *V. pleasii* (Marsh 1891)

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Abstract: Following the retreat of the last Pleistocene glaciers ~10,000 years before present, aquatic organisms re-colonized previously uninhabitable regions from various glacial refuges. Glaciations had major impacts shaping patterns of genetic diversity and population structure for organisms throughout North America. Knowledge of genetic population structure is critical for successful conservation programs involving an increasingly threatened freshwater fauna. Due to variations in life history and ecology, species-specific planning may be the most effective method for preserving rare or threatened species. The Ellipse mussel (*Venustaconcha ellipsiformis*) and its congener the Bleeding Tooth mussel (*V. pleasii*) are species of conservation concern through much of their respective ranges in the Midwestern United States. The Ellipse is found in small to medium rivers from the northern Ozark highlands north to the Upper Mississippi River drainage and into tributaries of Lake Michigan and Lake Huron. Mitochondrial DNA from the COI and ND1 regions was amplified to assess the genetic diversity and structure of these species. Phylogenetic analyses confirmed that *V. ellipsiformis* and *V. pleasii* are distinct species. Little variation was recovered in the Ellipse with a single common haplotype dominating throughout its range. For Ellipse, only limited genetic differentiation was found among the geographic regions sampled, with consistently significant differentiation only found between populations in the Illinois River drainage and populations in the northern Ozarks. The general low to moderate genetic structure among various geographically distant Ellipse populations suggests this species dispersed rapidly from unglaciated refugia with little time for genetic isolation to occur. The data suggest that *V. ellipsiformis* populations should be treated as three separate management units: northern Ozark highlands, Upper Mississippi River drainage, and the Illinois River/ Great Lakes drainages.

Key words: population genetics, Lampsilini, mtDNA, Ozark highlands, post-glacial colonization

The United States is home to the most diverse freshwater mussel fauna in the world (Lydeard *et al.* 2004, Graf and Cummings 2007, Bogan and Roe 2008). High levels of endemism occur in central and southeastern North America, making the region an important contributor to worldwide mussel diversity. However, due to a combination of habitat destruction and degradation associated with anthropogenic events, over 70% of the North American mussel fauna is now listed as endangered, threatened, or of special concern (Williams *et al.* 1993).

The Ellipse, *Venustaconcha ellipsiformis* (Conrad 1836), and Bleeding Tooth or Plea's Mussel, *V. pleasii* (Marsh 1891) are small (up to 75 mm), elliptical mussels that are generally found in swift currents of small to medium streams (van der Schalie and van der Schalie 1963, Oesch 1995). The Ellipse has a broad historical distribution in the central United States, ranging from Indiana and Michigan, west to Minnesota, and southwest to Oklahoma and Arkansas (Watters *et al.* 2009). Conversely, the Bleeding Tooth is a narrow range endemic of the White River drainage of the central Ozarks in Missouri and Arkansas (Oesch 1995, Riusech and Barnhart 2000). The Ellipse and Bleeding Tooth are considered species of conservation concern in many parts of their respective ranges (Williams *et al.* 1993).

Glochidia larvae of most freshwater mussels are obligate parasites on fish, and rely on this life stage for dispersal (Barnhart *et al.* 2008). Identified host fish for the Ellipse and Bleeding Tooth include several species of darters, sculpins, and the brook stickleback (Riusech and Barnhart 2000, Allen *et al.* 2007). These hosts all have relatively low vagility, which likely limits the dispersal abilities of both the host and parasite to relatively short distances (Woolnough *et al.* 2009). The inability to disperse long distances coupled with patchy distribution patterns likely reduces gene flow between populations, effectively isolating populations between drainages (Allen *et al.* 2007). Genetic drift may increase genetic differentiation between these populations (Hartl and Clark 2007). The level of genetic differentiation can, therefore, be viewed as a result of the competing forces of genetic drift increasing divergence and gene flow promoting homozygosity. Assessment of dispersal abilities and amount of gene flow between populations is then critical when attempting to preserve genetic diversity through conservation efforts.

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In addition to intrinsic effects related to individual dispersal abilities of a species and its host fish, historic geological changes have greatly affected genetic population structure in North American mussels. The primary geological event shaping modern day populations in the central and northern parts of North America was the Pleistocene glaciation (Larson and Schaetzl 2001). During this period, northern populations of aquatic organisms resided in multiple glacial refugia, isolating populations from one another (Soltis *et al.* 2006). Following the retreat of the last Pleistocene glaciers ~10,000 years before present, aquatic organisms used multiple dispersal routes to recolonize previously glaciated areas (Mandrak and Crossman 1992, Graf 2002). If individuals from a single glacial refuge colonized these newly colonized habitats, they likely experienced bottleneck effects and show reduced levels of genetic variation (Hewitt 1996). Therefore, higher genetic diversity can be expected in southern Ellipse populations or populations in unglaciated regions. Variation in population genetic structure between glaciated and unglaciated regions has been previously documented in several North American unionid species (Elderkin *et al.* 2007, 2008, Zanatta *et al.* 2007, Zanatta and Murphy 2008).

The factors influencing genetic variation in the North American mussel fauna makes it inadvisable to propose generalized propagation and conservation programs for unionids without *a priori* knowledge of evolutionary history and population genetics for the species in question (*e.g.*, Neves 2004). This is evidenced by the variation in genetic structure found among populations in the same watershed (*e.g.*, Grobler *et al.* 2006, Jones *et al.* 2006). Variation in dispersal abilities, life history, behaviors, and local adaptations to environmental conditions can significantly alter patterns of genetic diversity even in species with similar distributions (Elderkin *et al.* 2008).

Mitochondrial DNA (mtDNA) has long been used to assess patterns of phylogeography (Avise *et al.* 1987) and genetic structure in threatened aquatic species with respect to patterns of recent glacial history (Stepien and Faber 1998, Soltis *et al.* 2006, Elderkin *et al.* 2007, 2008, Zanatta and Murphy 2007, 2008). The main reason is that the mutation rate of mtDNA is much higher than that of nuclear DNA, allowing haplotype frequencies to drift and create genetic differences between populations in short periods of time (Beebee and Rowe 2008). This process produces significant genetic variation in most populations that are not extremely bottlenecked.

While information on the ecology, life history, and distribution of *Venustaconcha ellipsiformis* and *V. pleasii* are well established (van der Shalie and van der Schalie 1963, Riusech and Barnhart 2000, Allen *et al.* 2007), no research has documented the genetic diversity and population structure over their range. This study attempts to: (i) confirm species distinctions of *V. ellipsiformis* and its purported congener *V. pleasii*; (ii) resolve genetic relationships among populations across the range of the *V. ellipsiformis*; (iii) assess genetic diversity and differentiation among areas colonized following the Pleistocene glaciation and areas of probable glacial refuge; and (iv) put patterns of genetic variation and divergence in the context of conservation of these species.

MATERIALS AND METHODS

Sampling locations and collection

Specimens of *Venustaconcha ellipsiformis* and *V. pleasii* were collected from 16 waterbodies across the Midwestern U.S.A. *Venustaconcha ellipsiformis* specimens were collected from the following locations: Looking Glass River (Grand River/ Great Lakes drainage, Michigan (MI); 42.82228N, 84.69944W), Thornapple River (Grand River/ Great Lakes drainage, MI; 42.6114N, 85.0248W), Pine River (Saginaw River/ Great Lakes drainage, MI; 43.3212N, 84.7406W), Bourbeuse River (Meramec River drainage, Missouri (MO); 38.3799N, 91.0732W), Gasconade River, (MO; 38.3757N, 91.8272W), Buffalo Creek (Wapsipinicon River/ Upper Mississippi River drainage, Iowa (IA); 42.2061N, 91.4467W), Zumbro River (Upper Mississippi River drainage, Minnesota (MN); 44.0745N, 92.6634W), Salem Creek (Zumbro River/ Upper Mississippi River drainage, MN; 44.0705N, 92.8213W), Sugar Creek (Illinois River drainage, Indiana (IN); 40.6972N, 87.3817W), Mackinaw River (Illinois River drainage, Illinois (IL); 40.5711N, 88.6751W and 40.5542N, 88.5739W), Horse Creek (Illinois River drainage, IN; 41.1773N, 88.1468W), Ferson Creek (Fox River/ Illinois River drainage, IL; 41.9332N, 88.3411W), Forked Creek (Illinois River drainage, IL; 41.2552N, 88.1056W), Bray Creek (Illinois River drainage, IL; 40.5433N, 88.6267W) (Fig. 1). *Venustaconcha pleasii* specimens were collected from two locations: James River (White River drainage, MO; 37.2659N, 92.9501W) and Flat Creek (James River/ White River drainage, MO; 36.8022N, 93.7377W). Visual searches were conducted in riffle habitat from several tributary streams in each system, with 5–10 adult individuals being chosen for genetic analysis. Mantle tissue samples from each mussel were excised (Berg *et al.* 1995), therefore, only maternal mitotypes were recovered (Breton *et al.* 2007). Tissue samples were preserved in 95% ethanol in the field and stored at -80 °C upon return to the lab.

DNA extraction and genetic analysis

DNA was extracted from the individual samples using an overnight Proteinase K digestion of mantle tissue in cell lysis buffer followed by an alcohol extraction method (Sambrook

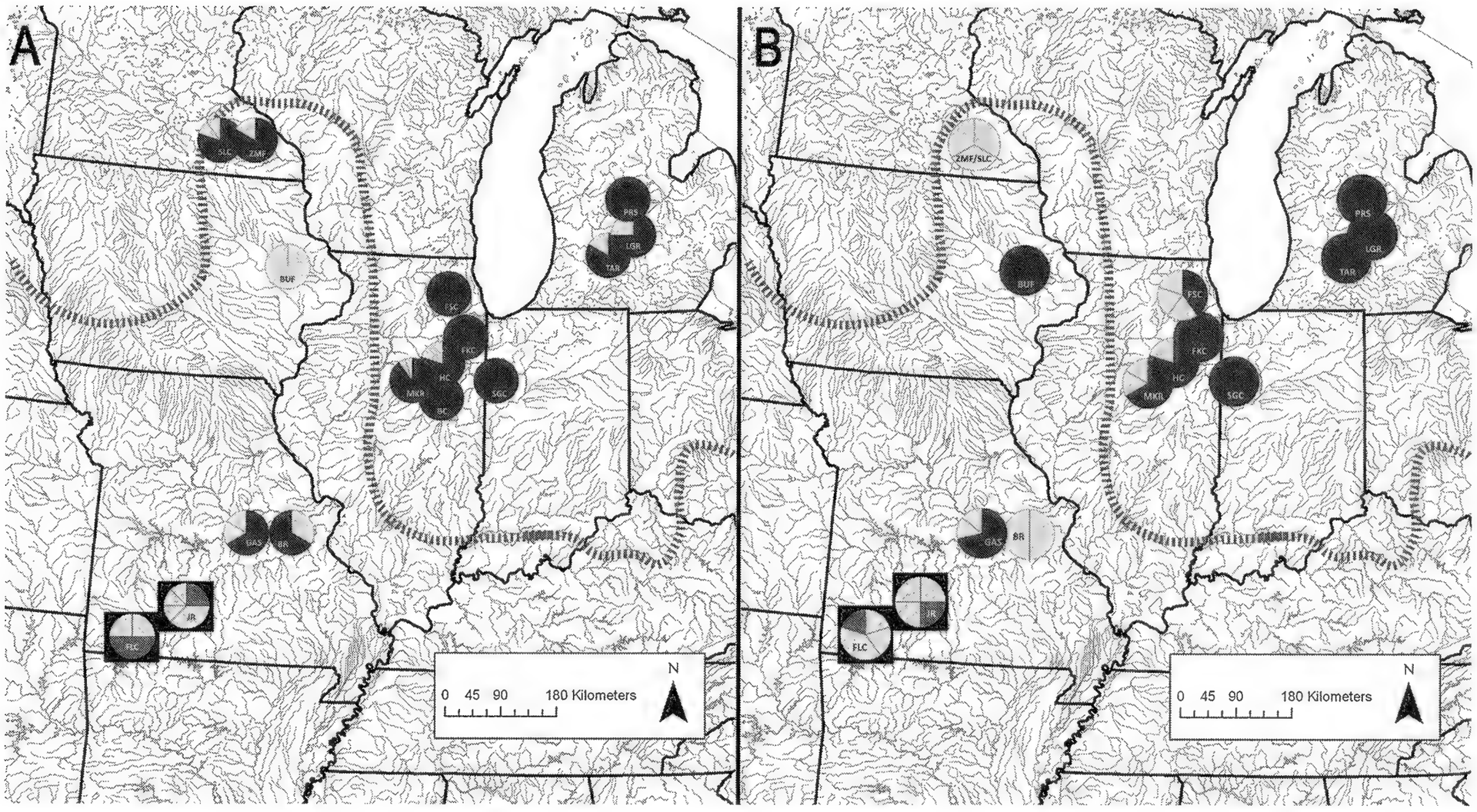

Figure 1. Sampling locations for *Venustaconcha ellipsiformis* (circles) and *V. pleasii* (squares) in the midwestern United States. The dashed line represents the approximate Wisconsin glacial maximum. Pie charts represent the frequency of (**A**) COI and (**B**) ND1 haplotypes encountered at each sampling location (Table 1). Common haplotypes (COI and ND1 haplotype 3 for *V. ellipsiformis* and COI haplotype 2 and ND1 haplotype 1 for *V. pleasii*) are shaded in dark grey. Uncommon and haplotypes unique to single populations (COI and ND1 haplotypes 1, 2, 4–13 for *V. ellipsiformis* and COI haplotypes 1, 3–7 and ND1 haplotypes 2–7) are shaded in light grey.

et al. 1989). Extracted genomic DNA was stained with SYBR Green™ and electrophoresed in a 1.5% agarose gel to confirm that genomic DNA had been properly extracted. Two mtDNA regions were amplified: cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1) using primers described in Campbell *et al.* (2005). Each reaction included 1 μL extracted DNA, 1 μL 10X PCR buffer, 1 μL bovine serum albumin, 0.3 μL of forward primer, 0.3 μL of reverse primer, 0.2 μL of dNTP, 0.05 μL *Taq* polymerase (Qiagen, Inc.), and deionized H_2O for a total reaction volume of 10 μL per sample. The thermocycler amplification conditions for both mtDNA regions were as follows: An initial heating of the sample to 92°C for 2 minutes; five cycles of 92°C for 40 seconds, 40°C for 40 seconds, and 72°C for 90 seconds; 25 cycles of 92°C for 40 seconds, 50°C for 40 seconds, and 72°C for 90 seconds; with a final elongation step 72°C for ten minutes and held at 4°C until they were placed in the freezer. Amplified PCR product was stained in SYBR Green and run on a 1.5% agarose gel electrophoresis to confirm successful amplification of the correct fragment length of DNA. The amplified samples were then purified using a QIAquick© PCR Purification Kit (Qiagen, Inc.). Amplified DNA was quantified using an ABI Nanodrop (Applied Biosystems, Inc.). The 5′ end of the amplified products were cycle-sequenced using 'Big Dye' Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Inc.) with the respective COI or ND1 forward primers (50 °C annealing temperature) and visualized on an ABI 3100 automated DNA sequencer (Applied Biosystems, Inc.) at the Michigan State University Research Technology Support Facility (RTSF) in East Lansing, Michigan.

Data analysis

Datasets for the sequenced genes were aligned using BIOEDIT (Hall 1999) and MACCLADE (Maddison and Maddison 1997) software. COLLAPSE v.1.2 (Posada 2004) was used to identify unique haplotypes from both *Venustaconcha ellipsiformis* and *V. pleasii* samples. Metrics for genetic diversity—number of haplotypes, number of polymorphic sites, and nucleotide diversity (π)—were calculated using ARLEQUIN v. 2.0 (Schneider *et al.* 2000) for each population sampled.

A maximum parsimony analysis was performed via a heuristic search with 1000 replications of random stepwise additions using PAUP* v. 4.0b10 (Swofford 1998). To gauge the robustness of nodes within the resulting trees, bootstrap values were calculated. Bootstrapping used 1000 replications and heuristic searching with ten random stepwise additions. Sequences of COI and ND1 sequences from *Epioblasma torulosa rangiana* (Lea 1838; COI-DQ479946,

ND1-DQ220720), *Lampsilis cardium* (Rafinesque 1820; COI-AF120653, ND1-GU085346), and *Pyganodon grandis* (Say 1829; COI-AF156504, ND1-GU085370) were used as outgroup taxa to root trees. PAUP* v. 4.0b10 was also used to calculate pairwise genetic distances among the haplotypes of *Venustaconcha ellipsiformis* and *V. pleasii.*

A second phylogenetic analysis used Bayesian inference implemented in MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001). The initial model of evolution was determined by comparing 24 models of evolution in MrModeltest 2.2 (Nylander 2004). MrBayes was run using 1,000,000 generations with 6 concurrently running Markov Chains and 2 hot chains, sampling every 100 generations.

TCS v. 1.21 (Clement *et al.* 2000) was used to construct a haplotype network based on the number of nucleotide mutations between different haplotypes. This program uses statistical parsimony to connect haplotypes based on a 95% confidence interval.

Hierarchical analyses of molecular variance (AMOVA) (Excoffier *et al.* 1992) was used to estimate the partitioning of haplotypes within and among populations. Because of limited numbers of samples taken from each sampling location, samples were pooled by major watershed/region (Great Lakes drainage, Northern Ozarks drainages, Illinois River drainage, and Upper Mississippi River drainage, and *Venustaconcha pleasii* in the White River drainage). An additional analysis was conducted to test genetic differentiation between glaciated and unglaciated sampling locations of *V. ellipsiformis*. AMOVAs for both COI and ND1 datasets were carried out in ARLEQUIN v. 2.0 by estimating Φ_{ST} from the absolute number of nucleotide differences and 16,000 permutations. To determine pooled sampling location differentiation, pairwise F_{ST} values were calculated using uncorrected *p*-distance (number of nucleotide substitutions). Finally, the number of haplotypes, number of polymorphic sites, and nucleotide diversity (π) were regressed against latitude of *V. ellipsiformis* sampling locations (of both *V. ellipsiformis* and *V. pleasii*) to test if geography and glaciation patterns could be determinate in assessing patterns of genetic diversity for these closely related species.

RESULTS

DNA sequencing consistently resulted in a 569 bp fragment for COI and 549 bp fragment for ND1. Because of the lack of sufficient overlap between samples successfully amplified, the COI and ND1 datasets could not be combined and were thus run separately in each analysis. A total of 13 distinct haplotypes were found from 101 *Venustaconcha ellipsiformis* COI sequences (Table 1; GenBank Accession numbers DQ220725 and KC537292–KC537304) and seven distinct haplotypes were found from nine COI sequences of *V. pleasii* (Table 2; GenBank Accession numbers KC537305–KC537311). A total of 13 distinct haplotypes were found from 56 *V. ellipsiformis* ND1 sequences, (Table 3; GenBank Accession numbers DQ220722 and KC537312–KC537323) and seven distinct haplotypes were found from 12 ND1 sequences of *V. pleasii* (Table 4; GenBank Accession numbers KC527324–KC537330). Metrics of genetic diversity by locus and population are in Tables 1 (COI) and 3 (ND1) for *V. ellipsiformis* and Tables 2 (COI) and 4 (ND1) for *V. pleasii.*

Phylogenetic analysis of the COI (Fig. 2) and ND1 loci (not shown) clearly distinguish *Venustaconcha ellipsiformis* and *V. pleasii* as distinct, monophyletic clades with high bootstrap support and Bayesian posterior probabilities confirming the status as two separate species. Average pairwise genetic distance between *V. ellipsiformis* and *V. pleasii* COI haplotypes was 4.31% (± 0.04% *SE*) and between ND1 haplotypes was 5.38% (± 0.08% *SE*). Haplotype networks were confirmed by the findings of the phylogenetic analysis (Fig. 3).

Venustaconcha ellipsiformis and *V. pleasii* formed separate networks of closely related haplotypes. *Venustaconcha ellipsiformis* populations had on average 1.8 haplotypes per population for COI and 1.9 haplotypes per population for ND1. Populations were mostly dominated by a single common haplotype (h3 for both COI and ND1, Tables 1 and 3, Fig. 3). These two common haplotypes comprised 85.1% of all COI sequences and 69.6% of all ND1 sequences (Table 1 and 3). These haplotypes were recovered from 92.8% (COI) and 83.3% (ND1) of sampling locations (Table 1 and 3, Fig. 1). The more limited sampling of *V. pleasii*, resulted in one shared haplotype for both COI and ND1 between the two sampling locations, however these haplotypes did not dominate either population (Table 2 and 4, Fig. 1).

Significant population structure was evident using COI and ND1 sequences in *Venustaconcha ellipsiformis.* The AMOVA for COI and ND1 respectively indicated that 7.53% (COI; $\Phi_{ST} = 0.075$, $p = 0.028$) and 6.88% (ND1, $\Phi_{ST} = 0.069$, $p < 0.001$) of the variation resided within *V. ellipsiformis* populations, whereas 92.47% (COI) and 93.12% (ND1) occurred among populations. Based on F_{ST} for COI, significant differences ($p < 0.05$) were only observed between pooled population samples in the Illinois River drainage and the Upper Mississippi River and the Illinois River drainage and the Northern Ozarks (Table 5). A somewhat different pattern among pooled populations in pairwise F_{ST} was apparent for ND1 with significant differences ($p < 0.05$) were observed between the Great Lakes drainage and the Upper Mississippi River and between the Illinois River drainage and the Northern

Table 1. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for COI in 14 populations of *Venustaconcha ellipsiformis*. Sampling locations are: Ferson Creek (FSC), Forked Creek (FKC), Horse Creek (HC), Mackinaw (MKR), and Bray Creek (BRC), Illinois; Sugar Creek (SGC), Indiana; Buffalo Creek (BFC), Iowa; Pine River (Saginaw River drainage; PRS), Looking Glass River (LGR), and Thornapple River (TAR), Michigan; Zumbro River (ZMF) and Salem Creek (SLC), Minnesota; Gasconade River (GAS), Bourbeuse River (BR), Missouri.

Haplotypes and polymorphic nucleotide sites																									Populations														
																									Great Lakes			Northern Ozarks		Upper Mississippi			Illinois River drainage						
																									T	P	L		G	B	Z	S	S	M		F	F	
							1	1	1	1	2	2	2	2	2	2	3	4	4	4	5	5			A	R	G	B	A	U	M	L	G	K	H	S	K	B
			1	1	1	2	0	6	6	7	1	1	3	4	6	9	3	2	5	6	4	7			R	S	R	R	S	F	F	C	C	R	C	C	C	C
	6	9	1	2	3	8	8	0	9	1	2	3	9	6	1	9	3	1	8	5	3	0			*n*=6	*n*=6	*n*=4	*n*=6	*n*=6	*n*=2	*n*=6	*n*=10	*n*=6	*n*=20	*n*=10	*n*=10	*n*=4	*n*=5
1	C	G	T	G	T	T	T	T	T	G	G	C	G	G	A	T	T	T	T	A	A	C			-	-	-	-	-	2	-	-	-	-	-	-	-	-
2	*	*	*	*	*	*	*	*	*	A	*	*	*	*	G	*	*	*	*	*	G	T			-	-	-	2	-	-	-	-	-	-	-	-	-	-
3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			5	6	3	4	4	-	5	8	6	18	8	10	4	5
4	*	*	*	*	G	*	*	A	A	*	T	*	T	*	*	C	A	A	*	*	G	*			-	-	-	-	1	-	-	-	-	-	-	-	-	-
5	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	G	*			-	-	-	-	1	-	-	-	-	-	-	-	-	-
6	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			-	-	-	-	-	-	-	-	-	-	2	-	-	-
7	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	C	*	G	*			-	-	1	-	-	-	-	-	-	-	-	-	-	-
8	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	G	*			-	-	-	-	-	-	-	-	-	1	-	-	-	-
9	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			-	-	-	-	-	-	-	-	-	1	-	-	-	-
10	*	T	G	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			-	-	-	-	-	-	-	1	-	-	-	-	-	-
11	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	G	*			-	-	-	-	-	-	-	1	-	-	-	-	-	-
12	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	G	*			1	-	-	-	-	-	-	-	-	-	-	-	-	-
13	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*			-	-	-	-	-	-	1	-	-	-	-	-	-	-
																																						-
Number of haplotypes:																									2	1	2	2	3	1	2	3	1	3	2	1	1	1
Number of polymorphic sites:																									1	0	2	3	9	0	1	4	0	2	1	0	0	0
Nucleotide diversity (π) per population:																									0.0006	0	0.0018	0.0035	0.0053	0	0.0006	0.0004	0	0.0004	0.0006	0	0	0
± Standard deviation:																									0.0008		0.0017	0.0029	0.0037		0.0008	0.0005		0.0005	0.0007			

Table 2. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for COI in 2 populations of *Venustaconcha pleasii*. Sampling locations are: Flat Creek (FLC) and James River (JR), Missouri.

Haplotypes and polymorphic nucleotide sites									Populations	
									F	
	1	2	2	3	4	5			L	J
	3	3	1	2	6	0	9	5	C	R
	3	9	4	6	1	0	6	9	$n = 4$	$n = 8$
1	T	A	G	G	G	A	T	T	1	-
2	*	*	*	*	*	G	*	*	2	2
3	C	*	*	*	*	G	C	*	1	-
4	*	*	*	*	A	G	*	*	-	3
5	*	G	*	*	*	G	*	*	-	1
6	*	*	*	A	*	G	C	*	-	1
7	*	*	A	*	*	G	*	C	-	1
Number of haplotypes:									3	5
Number of polymorphic sites:									3	6
Nucleotide diversity (π) per population:									0.0026	0.0031
± Standard deviation:									0.0024	0.0023

Ozarks drainages (Table 5). For both loci, *V. pleasii* was strongly divergent from all *V. ellipsiformis* populations (Table 5).

Evidence of effects of glaciation on genetic diversity was observed. AMOVA recovered significant F_{ST} values between glaciated and unglaciated regions, however F_{ST} values were in the low to moderate range (Wright 1978; Table 6). Only *Venustaconcha ellipsiformis* ND1 nucleotide diversity showed a significant correlation with latitude ($R^2 = 0.405$, $p = 0.014$).

Discussion

Within-population structure

Most of the genetic variation in *Venustaconcha ellipsiformis* and *V. pleasii*, is found within populations. Average haplotype diversity of these species is but similar to sympatrically occurring unionids found in small to medium rivers (*e.g.*, *Epioblasma triquetra* (Rafinesque, 1820) (Zanatta and Murphy 2008) and *Elliptio dilatata* (Rafinesque 1820) (Elderkin *et al.* 2008)).

The pattern of population genetic variation found in the Ellipse and Bleeding Tooth mussels is largely congruent with sympatrically occurring unionids and fishes. The Pleistocene glaciations had major impacts on the genetic structure of organisms in North America (Soltis *et al.* 2006), including unionids and their hosts (*e.g.*, Elderkin *et al.* 2007, 2008, Zanatta and Murphy 2007, 2008). During the repeated advances of Pleistocene glaciers, aquatic species were forced to retreat into one or more glacial refugia causing the loss of within-population diversity due to genetic drift and possible bottlenecks (Hewitt 1996). As the glaciers receded, fishes and their associated unionid parasites dispersed from these refugia and recolonized suitable habitats (Mayden 1988, Mandrak and Crossman 1992, Graf 2002). The newly founded populations tended to have reduced genetic diversity as they were most probably colonized by a small number of individuals (Hewitt 1996). *Venustaconcha ellipsiformis* and *V. pleasii* follow the expected pattern with populations in unglaciated regions showing more genetic diversity than more northerly regions unaffected by the Pleistocene glaciers. A strong pattern of fewer haplotypes per population, lower within-population nucleotide diversity, and fewer polymorphic sites per population as latitude increases was observed in the analyses of *V. ellipsiformis* and *V. pleasii* populations. Furthermore, *V. ellipsiformis* populations in formerly glaciated habitats showed low to moderate, but statistically significant genetic divergence from populations in unglaciated regions. As expected, populations of *V. ellipsiformis* in recently glaciated Great Lakes and Illinois River drainages show markedly lower genetic diversity than *V. ellipsiformis* and *V. pleasii* in unglaciated Ozark highlands. This indicates that repeated glaciations in the modern range of *V. ellipsiformis* may have resulted in a genetic bottleneck, whereas *V. pleasii* was not bottlenecked as populations were not directly affected by glaciations. Furthermore, the more northerly populations in recently glaciated watersheds may have further reduced genetic diversity because of a founder effect following the most recent glacial retreat.

Among-population structure

Phylogenetic analyses gave strong support for *Venustaconcha ellipsiformis* and *V. pleasii* as distinct sibling species. Pairwise genetic distance between these species is similar to values observed among species in the genus *Epioblasma* (Rafinesque 1831; Jones *et al.* 2006). Genetic data are further supported by the range disjunctions between the two species, differential host usage (Riusech and Barnhart 2000), and differing shell morphologies (Oesch 1995). The existence of distinct clades for species and species complexes in the various drainages of the central highlands (Ozark and Ouachita mountains) has been shown for fish species (Near *et al.* 2001, Berendzen *et al.* 2010) including confirmed hosts for *V. ellipsiformis* and *V. pleasii* (Ray *et al.* 2006). Based on the phylogeographic pattern revealed in other unionids in central North America (Grobler *et al.* 2006, Elderkin *et al.* 2007, Elderkin *et al.* 2008), additional specimens from the *Venustaconcha* Thiele, 1934 species

Table 3. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for ND1 in 12 populations of *Venustaconcha ellipsiformis*. Sampling locations are as in Table 1.

Haplotypes and polymorphic nucleotide sites																			Populations											
																			Great Lakes			Northern Ozarks		Upper Mississippi		Illinois River drainage					
					1	2	2	2	2	3	4	4	4	4	4	5	5	5	T	P	L		G	B	Z	S	M		F	F
	3	5	5	8	6	0	0	4	5	1	1	7	8	8	9	1	2	3	A	R	G	B	A	U	M	G	K	H	S	K
	3	6	7	1	0	5	7	7	2	8	0	2	0	3	4	6	5	0	R	S	R	R	S	F	F	C	R	C	C	C
																			n=6	*n*=4	*n*=2	*n*=6	*n*=7	*n*=2	*n*=3	*n*=6	*n*=6	*n*=5	*n*=5	*n*=4
1	G	G	T	G	G	T	T	T	A	G	A	T	G	G	A	G	G	A	-	-	-	3	-	-	-	-	-	-	-	-
2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	-	-	-	3	-	-	-	-	-	-	-	-
3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	6	4	2	-	5	2	-	6	4	4	2	4
4	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	G	-	-	-	-	-	-	-	-	-	-	1	-
5	T	*	*	*	*	*	*	*	*	*	T	*	A	*	*	*	C	G	-	-	-	-	-	-	-	-	-	-	1	-
6	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	T	G	-	-	-	-	-	-	-	-	1	-	1	-
7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	-	-	-	-	1	-	-	-	-	-	-	-
8	*	T	A	*	T	A	A	A	T	A	*	A	*	*	*	*	*	G	-	-	-	-	1	-	-	-	-	-	-	-
9	*	*	*	A	*	*	*	*	*	*	*	*	*	A	*	*	*	G	-	-	-	-	-	-	-	-	-	1	-	-
10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	G	-	-	-	-	-	-	-	-	1	-	-	-
11	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	-	-	-	-	-	-	1	-	-	-	-	-
12	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	-	-	-	-	-	-	1	-	-	-	-	-
13	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	G	-	-	-	-	-	-	1	-	-	-	-	-
Number of haplotypes:																			1	1	1	2	3	1	3	1	3	2	4	1
Number of polymorphic sites:																			0	0	0	1	9	0	3	0	2	2	4	0
Nucleotide diversity (π) per population:																			0	0	0	0.0033	0.0063	0	0.0049	0	0.0012	0.0007	0.0031	0
± Standard deviation:																						0.0025	0.0042		0.0044		0.0012	0.0009	0.0025	

Table 4. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for ND1 in 2 populations of *Venustaconcha pleasii*. Sampling locations are as in Table 1.

Haplotypes and polymorphic nucleotide sites															Populations	
															F	
	1	1	1	1	2	3	3	3	3	3	4	5	5		L	J
6	2	4	4	4	8	0	4	5	6	8	9	1	3		C	R
	9	5	0	4	9	1	5	1	9	8	9	3	5	6	$n=5$	$n=4$
1	C	T	T	T	T	T	T	T	T	C	A	G	A	C	1	1
2	*	C	*	C	*	*	*	*	*	*	*	*	*	*	1	-
3	T	C	*	C	*	C	*	C	*	T	G	*	G	*	2	-
4	T	C	*	C	*	C	C	C	*	*	G	*	G	*	1	-
5	T	C	C	C	*	C	*	C	*	T	G	*	G	A	-	1
6	T	C	C	C	*	C	*	C	*	T	G	*	G	A	-	1
7	T	C	*	C	C	C	*	C	C	T	G	T	G	*	-	1
Number of haplotypes:															4	4
Number of polymorphic sites:															9	14
Nucleotide diversity (π) per population:															0.0088	0.0135
± Standard deviation:															0.0060	0.0095

complex should be considered in a phylogeographic context. These include probable *V. pleasii* from the Neosho/Arkansas River drainage and newly revealed putative members of the *Venustaconcha* clade: *Villosa constricta* (Conrad, 1838; mid-Atlantic drainages), *Villosa perpurpurea* (Lea, 1861; Upper Tennessee River drainages), and *Villosa trabalis* (Conrad, 1834; Upper Tennessee and Cumberland River drainages) (Kuehnl 2009).

Significant regional genetic structure was found among *Venustaconcha ellipsformis* populations. The differences between the analyses of the COI and ND1 datasets likely resulted from limited sample sizes in the ND1 dataset. Low to moderate (and statistically significant) levels of genetic divergence were found among populations in the Great Lakes/Illinois River (glaciated) and the northern Ozarks and the Upper Mississippi populations (non-glaciated). Low levels of divergence were also found between the Upper Mississippi and the Northern Ozarks (significant only using ND1); all non-glaciated regions. The divergence found between the unglaciated Upper Mississippi and Northern Ozarks populations of *V. ellipsiformis* is congruent with a hypothesized northern glacial refuge in the "Driftless Area" of the upper Midwest (Rowe *et al.* 2004). Furthermore, as in Rowe *et al.* (2004), our data largely fail to meet expectations for a northward expansion out of a southern refugium, with genetic diversity showing little to no gradient from south to north. Divergence was not significant between the Great Lakes and the Illinois River; all in formerly glaciated regions, giving further support for these populations arising from a single glacial refuge. However, limited evidence of a south-to-north pattern of decreasing genetic diversity moving out of glacial refuges is consistent with multiple glacial refugia and rapid colonization of newly deglaciated waterways. The low to moderate levels of genetic divergence are also expected given the limited time for isolation and genetic drift to have an effect on structure (Freeland 2005). Furthermore, our results are consistent with the pattern of post-glacial colonization into the central Great Lakes region hypothesized for *V. ellipsiformis* by van der Schalie and van der Schalie (1963).

The results of this study are broadly congruent with other recent phylogeographic studies on unionids. The patterns of genetic structure observed in *Elliptio dilatata* (Elderkin *et al.* 2008) and *Epioblasma triquetra* (Zanatta and Murphy 2008) are the best for direct comparison to that of *Venustaconcha ellipsiformis* because these species share similar small-medium river habitats and have somewhat overlapping distributions. For *E. dilatata*, low to moderate levels of differentiation were found among populations in previously glaciated regions (Great Lakes and Ohio basins), but unglaciated regions were highly divergent from populations in the central highlands in the White River and Ouachita River drainages (Elderkin *et al.* 2008). Similarly in *E. triquetra*, using COI sequence data, high levels of divergence were found between populations from the southern slope of the Ozarks (St. Francis River) and previously glaciated regions, but low levels among the northern slope of the Ozarks (Bourbeuse River) and previously glaciated regions (Zanatta and Murphy 2008). The patterns observed for *V. ellipsiformis* and *V. pleasii* are somewhat puzzling in that they are not known from the Ohio River drainage (Watters *et al.* 2009). Furthermore, specimens from the Illinois River and Lake Michigan drainages were not considered in studies on *E. dilatata* or *E. triquetra*.

The relatively low levels of genetic differentiation observed among Ellipse populations can be linked to the phylogeography and population structure of sympatrically occurring fish species – some of which are probable glochidial hosts. The phylogeographic pattern of a parasitic organism and its host should be broadly congruent and has been shown to be so in unionids (Zanatta and Wilson 2011). Several darter species of *Etheostoma* and *Percina* and sculpins (*Cottus*) are confirmed hosts for the Ellipse and Bleeding Tooth (Ruisech and Barnhart 2000, Allen *et al.* 2007). The distribution of the rainbow darter, *Etheostoma caeruleum* (Storer 1835), a confirmed host for both Ellipse and Bleeding Tooth (Ruisech and Barnhart 2000, Allen *et al.* 2007), is much broader that of the Ellipse and Bleeding Tooth, but the patterns of phylogeography and population structure

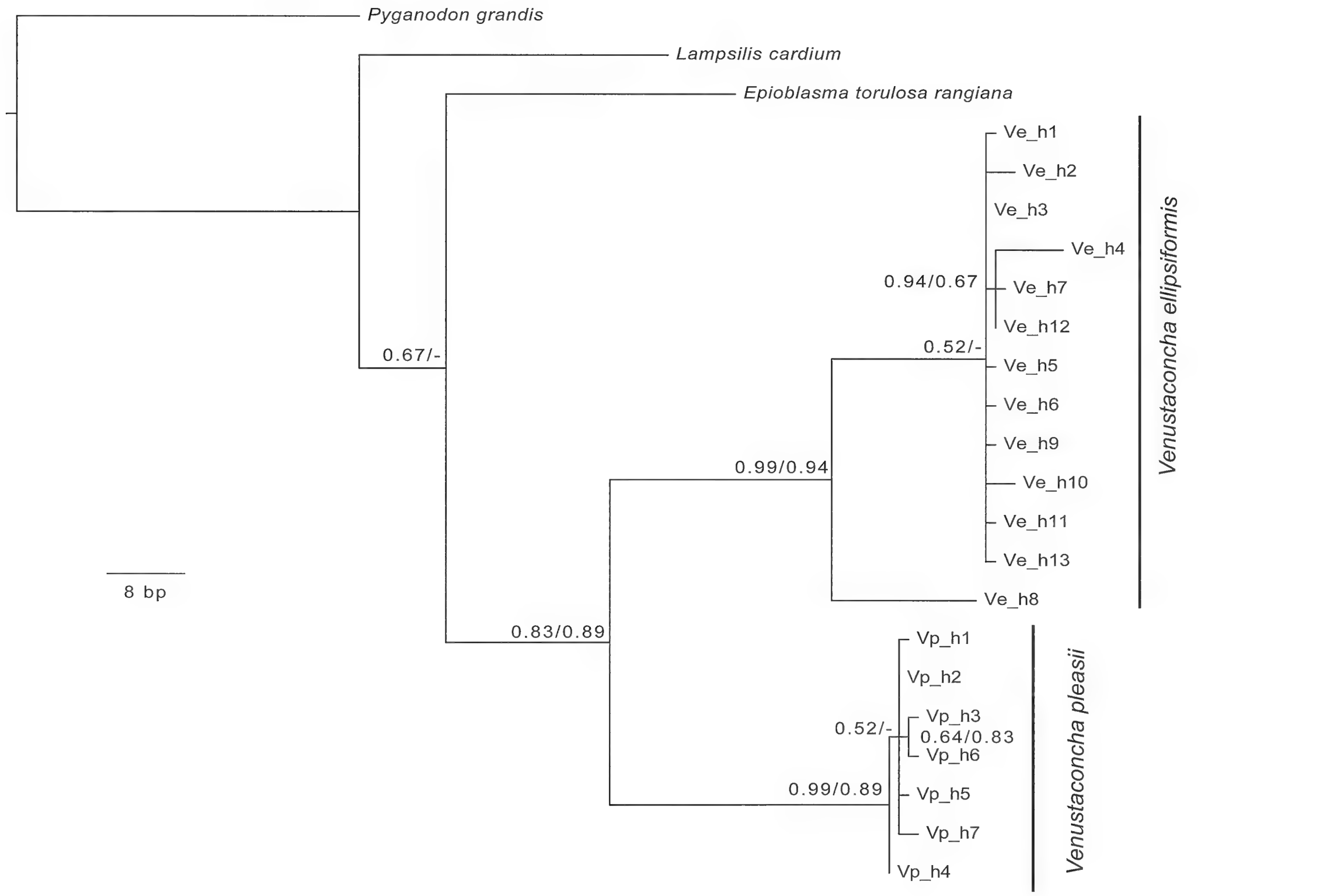

Figure 2. Phylogram of *Venustaconcha ellipsiformis* (Ve) and *V. pleasii* (Vp) from 16 populations in the United States Midwest. *Epioblasma torulosa rangiana*, *Lampsilis cardium*, and *Pyganodon grandis* were used as outgroups and the tree was rooted with *Pyganodon grandis*. The numbers above the branches and before the slash indicate the proportion of bootstrap replicates where the clade was found under a maximum parsimony framework. The numbers after the slash are the calculated posterior probabilities (greater than 50%), indicating the proportion of trees that these nodes appeared in the Bayesian topology.

of the rainbow darter in the Ozark highlands and Upper Mississippi River appears to closely match that of the Ellipse and Bleeding Tooth (Ray *et al.* 2006). The congruency of pattern breaks down in the Illinois River and Great Lakes drainages, as rainbow darters in this region belong to a clade that appears to have invaded the region via the Ohio and Wabash River systems, where the Ellipse and Bleeding Tooth are not present. Two other sympatrically occurring species with Ellipse and Bleeding Tooth, the gilt darter, *Percina evides* (Jordan and Copeland 1877), and Ozark minnow, *Notropis nubilus* (Forbes 1878), also show a similar phylogeographic patterns between the Ozarks and Upper Mississippi systems (Near *et al.* 2001, Berendzen *et al.* 2010), but neither of these species have distributions that extend into the Illinois River or Great Lakes drainages. Each of these fish species showed low to moderate levels of genetic differentiation among populations in northern Ozark streams (*i.e.*, Gasconade, Osage, Meramec river systems) and the Upper Mississippi drainage, similar to the patterns observed for the Ellipse mussel.

Genotyping of microsatellite DNA markers have proven useful in determining fine-scale genetic structure and recent differentiation among unionid populations (*e.g.*, Kelly and Rhymer 2005, Jones *et al.* 2006, Zanatta *et al.* 2007), typically revealing more resolution than mtDNA sequence data. Additional study of these Ellipse and Bleeding Tooth populations using microsatellite markers is recommended. Amplifying and optimizing published microsatellite loci for other lampsiline mussels has been attempted for *Venustaconcha ellipsiformis* (Eackles and King 2002, Jones *et al.* 2004, Zanatta and Murphy 2006), but only two of these loci (LabD111 and LabC23; Eackles and King 2002) consistently amplified and were polymorphic in populations from Michigan (Zanatta, unpublished data). Additional

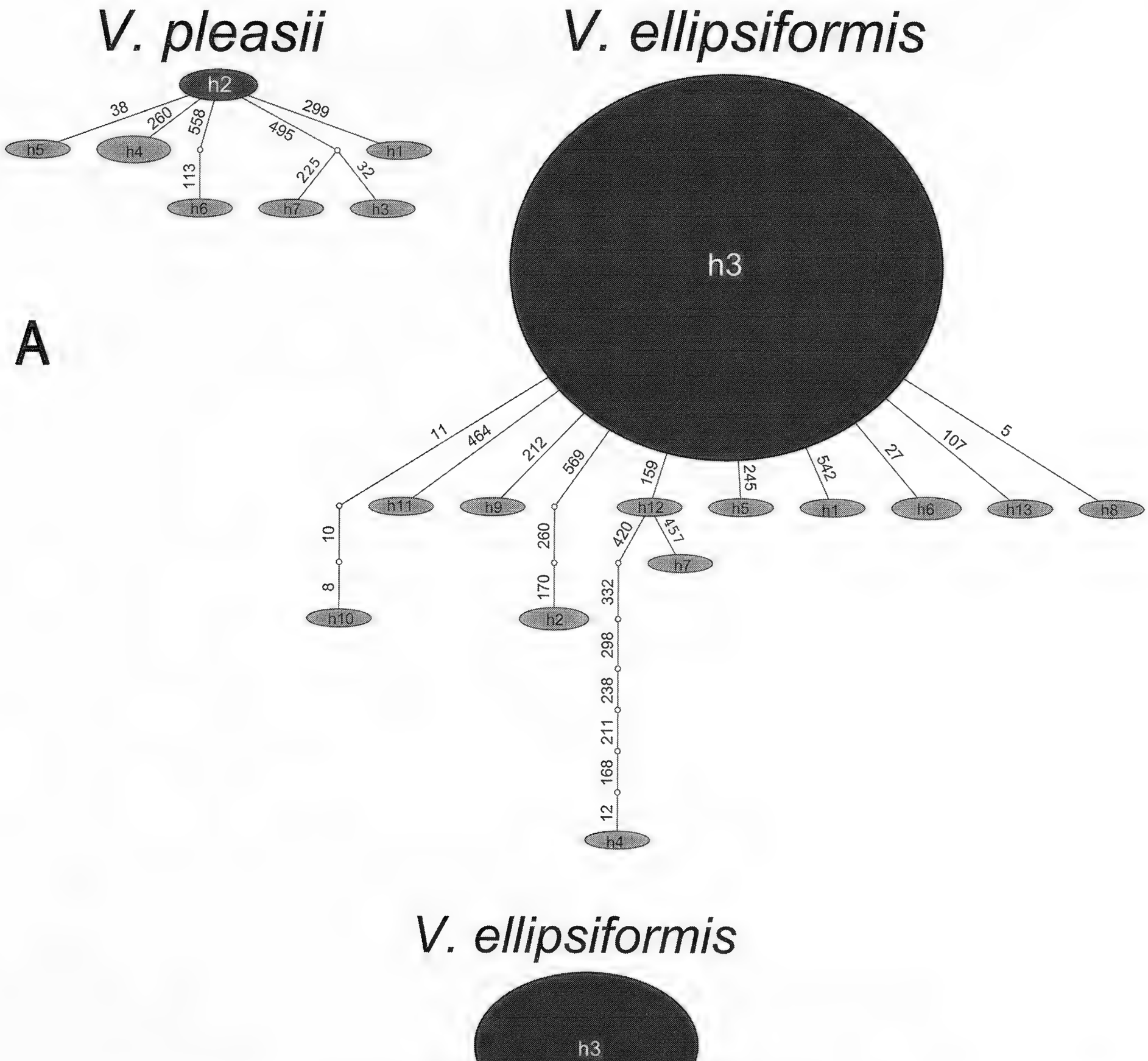

Figure 3. Spanning networks of mtDNA haplotypes at the (**A**) COI and (**B**) ND1 locus for *Venustaconcha ellipsiformis* and *V. pleasii*. Connecting lines represent a single base pair difference between two haplotypes and numbers between each node represents location of the base pair change. Sizes of the circles correspond to the frequency of haplotypes encountered, and small nodes represent intermediate haplotypes not encountered in sampling. Dark grey circles represent common shared haplotypes.

Table 5. Pairwise F_{ST} values among four pooled populations of *Venustaconcha ellipsiformis* and *V. pleasii* from ND1 (below diagonal) and COI (above diagonal). Values in bold are statistically significant ($\alpha = 0.05$).

	Great Lakes drainage	Northern Ozarks drainages	Upper Mississippi drainage	Illinois River drainage	*V. pleasii* White R. drainage
Great Lakes drainage	-	0.043 ($p = 0.113$)	0.023 ($p = 0.172$)	0.068 ($p = 0.068$)	**0.959 ($p < 0.001$)**
Northern Ozarks drainages	0.063 ($p = 0.053$)	-	**0.051 ($p = 0.031$)**	**0.187 ($p = 0.002$)**	**0.912 ($p < 0.001$)**
Upper Mississippi drainage	**0.189 ($p = 0.015$)**	-0.014 ($p = 0.490$)	-	**0.061 ($p = 0.006$)**	**0.950 ($p < 0.001$)**
Illinois River drainage	-0.011 ($p = 0.663$)	**0.111 ($p < 0.001$)**	0.087 ($p = 0.124$)	-	**0.981 ($p < 0.001$)**
V. pleasii White R. drainage	**0.910 ($p < 0.001$)**	**0.851 ($p < 0.001$)**	**0.846 ($p < 0.001$)**	**0.930 ($p < 0.001$)**	-

markers specifically designed for *Venustaconcha* will need development before future fine-scale genetic analyses can be conducted.

Conservation implications and summary

This study confirms *Venustaconcha ellipsiformis* and *V. pleasii* are distinct species. Low to moderate levels of genetic structure was found among populations in Upper Mississippi and across the Chicago outlet between Great Lakes and Illinois River. In the interest of conservation and recovery of populations, *V. ellipsiformis* populations appear to be generally compatible within recently glaciated regions. Based on the evidence presented herein and using a cautionary approach, there is partial support (mostly from COI dataset) for three management units (Moritz 1994, Fraser and Bernatchez 2001) in *V. ellipsiformis*: (1) northern Ozarks (2) Upper Mississippi (3) Illinois River and Great Lakes. Following previously published guidelines, these management units should be taken into consideration when planning future conservation, propagation and population augmentation programs for *V. ellipsiformis* (Neves 2004, Jones *et al.* 2006). It is recommended that additional sampling and fine-scale population genetic analysis be conducted with additional individuals from the southern part of the range of *V. ellipsiformis*, including the upper Arkansas River drainage. The findings of this study should be further tested at a finer scale with microsatellite DNA markers.

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Table 6. Pairwise F_{ST} values among four pooled populations of *Venustaconcha ellipsiformis* and *V. pleasii* from ND1 (below diagonal) and COI (above diagonal) in glaciated and unglaciated regions. Values in bold are statistically significant ($\alpha = 0.05$).

	Glaciated Sites: Great Lakes + Illinois R. drainages	Unglaciated Sites: Northern Ozarks + Upper Mississippi R. drainages	*V. pleasii* White R. drainage
Glaciated Sites: Great Lakes + Illinois R. drainages	-	**0.035 ($p = 0.002$)**	**0.982 ($p < 0.001$)**
Unglaciated Sites: Northern Ozarks + Upper Mississippi R. drainages	**0.086 ($p < 0.001$)**	-	**0.934 ($p < 0.001$)**
V. pleasii White R. drainage	**0.947 ($p < 0.001$)**	**0.867 ($p < 0.001$)**	-

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A stable isotope tracer (δ^{13}C) study of *Escherichia coli* retention in two freshwater bivalves (*Corbicula fluminea* and *Elliptio complanata*) (Corbiculidae and Unionidae)

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Abstract: Bacteria are ingested by suspension feeding bivalves and can be an important component of their diet. This study evaluated whether a common bacterium of vertebrate enteric origin, *Escherichia coli* (Migula, 1895), is retained in the stomach or gill by two different freshwater bivalve species, *Corbicula fluminea* (Müller, 1974) and *Elliptio complanata* (Lightfoot, 1786). A series of diet treatment experiments were conducted comparing each anatomical section using a δ^{13}C label and *E. coli* cells grown from stock cultures. A significant difference in δ^{13}C values was related to anatomic structure among all treatment groups during a 7-day feeding experiment (24 jars, $df = 140$; $F = 4.88$; $P < 0.001$). A key finding was that in gill tissue, a significant difference was observed among δ^{13}C-labeled and unlabeled treatment combinations for both *C. fluminea* and *E. complanata* ($F = 13.57$; $df = 31$; $P < 0.0001$). The results suggest that water column *E. coli* are likely retained on gill tissue and to a lesser degree in the stomach in both *E. complanata* and *C. fluminea*. This study serves to validate the hypothesis that *E. coli* may be initially more abundant in gill tissue during sorting processes before being transferred to the stomach.

Key words: bacterial feeding and retention, freshwater mollusks

Ambient bacteria in aquatic systems are considered a potential dietary nutrient resource for bivalves (ZoBell and Feltham 1938, Langdon and Newell 1990). Bacteria are thought to contribute to the diets of both freshwater and marine bivalves (Al Jebouri and Trollope 1984, Wen *et al.* 2009). Bacteria may be serving as a dietary resource or contributing to indigenous flora essential for digestion. Stable isotope studies have documented the constituent assimilation of bacteria-derived nitrogen and carbon in freshwater mussel (Unionidae) tissues (Nichols and Garling 2000). Changes in bacterial communities associated with aquatic habitat degradation (Williams *et al.* 1993, Baker and Levinton 2003) could potentially be contributing to the marked declines in freshwater bivalve populations that have been documented during the past 20 years (Bogan 1993). The diets of many marine bivalves have been continually refined during the last 20 years to support their commercial production (Langdon and Onal 1999, Dame 2011) and bacteria are used as a probiotic to support larval health (Lim *et al.* 2011). Efforts to propagate freshwater mussels (unionids) to support conservation and augmentation of remaining populations have also prompted studies to refine the diets of these freshwater bivalves (Riera *et al.* 2000, Piola *et al.* 2006) and bacteria may play an important role in the health and growth of unionids in captivity.

The microbiota of bivalve mollusks has been examined mainly from a public health perspective, since they may concentrate pathogenic microorganisms (*i.e.*, *Escherichia coli*, *Vibrio* spp.) and pose a food safety problem (Bernard 1989, Newell 2004). Although the tissues that retain bacteria in marine bivalves are well documented (Decho and Luoma 1991, Shumway 1992, De Leon and Jaykus 1997), we know little about what anatomic structures in freshwater unionids retain bacteria. Retention is initiated when particles are sorted by gill cilia. The particles are then transferred to the stomach including the remainder of the digestive tract. Retention is described as the accumulation of water column bacteria onto internal anatomical structures prior to ingestion in the digestive tract. Ingested particles enter the stomach where they are subject to mechanical disruption by the crystalline style and enzymatic degradation (Bayne *et al.* 1976).

Bacterial populations in aquatic systems are numerous and diverse and include species of variable relevance to freshwater bivalve diets. Species of *Escherichia coli*, *Pseudomonas* and *Vibrio* that contribute to these bacterial assemblages can dominate the microbiota in bivalve habitats (Pujalte *et al.* 1999, Vaughn *et al.* 2008). Those species not useful as a nutrient resource, however, may competitively inhibit adherence and retention of bacteria (Mevel *et al.* 1990). Active competitive inhibition between different species of bacteria may adversely affect the health, survival and abundance of freshwater bivalve populations (Sockett *et al.* 1985). Freshwater

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bivalves such as Unionidae and *Corbicula fluminea* (Müller, 1974) sort different-sized bacteria before the particles enter the mouth (Morton 1983, Silverman *et al.* 1997). Latero-frontal internal cirri of the gills of some species play a prominent role in the sorting process (Morton 1983). There appears to be a correlation between their ability to capture smaller particles such as bacteria (< 1 μm) and complex cirral structure and clearance rate (Ward and Shumway 2004).

The objective of this study was to determine whether two freshwater bivalves retain a common bacterium (*Escherichia coli*) present in nutrient polluted waters. We examined evidence of whether *E. coli* is retained by external (*i.e.*, gills) and internal (*i.e.*, stomach) anatomical structures of a native species, *Elliptio complanata* (Lightfoot, 1786), and a non-native species, *Corbicula fluminea*.

MATERIALS AND METHODS

Bacteria cultivation and stable isotope labeling

Escherichia coli used in the feeding experiments were a non-pathogenic laboratory strain (#25922) adapted for microbial cloning and previously stored at -80 °C. A culture and labeling procedure followed. A loop of the isolate was directly inoculated from frozen stock into a standard culture nutrient media (Luria-Bertani [LB], Becton Dickinson, Sparks, Maryland) for propagation of *E. coli* cells. The culture media was then incubated at 37 °C for 24 hours. Cultivation of non-isotopically labeled bacteria culture was then used to prepare δ^{13}C-labeled bacteria and experimental diet sources.

Stable isotope compounds can be used as a tracer label to distinguish between food sources of bacteria. The content of the heavy carbon isotope ^{13}C is a common tracer and expressed as the atom percent or ratio (R) of the amount of the rare isotope to the abundant isotope (*e.g.*, $R = {}^{13}C/{}^{12}C$). The natural abundance of the rare isotope is low, and the ratio of the numerically small stable isotope is expressed in "delta" notation as values in parts per thousand (per mil or ‰), where δ ‰ = $(R_A/R_{Std} - 1)$ x 1,000‰, and R_A and R_{Std} as molar ratios of the rare isotope to the abundant isotope (*e.g.*, $^{13}C/^{12}C$) in the sample and an internationally recognized standard, respectively. Isotope (δ^{13}C) labeled cultures were prepared by combining a δ^{13}C label with non-isotopically labeled *E. coli* cells. Specifically, the experimental *E. coli* were labeled by growing cells in D-glucose $^{13}C_6$® (Dg-^{13}C) LB media in solution with non-isotopically labeled bacteria cells. Populations were harvested after incubation at the peak (logarithmic) growth phase (24–48 hours). A NanoDrop® fluorospectrometer (Thermo Scientific, Wilmington, Delaware) was used to measure adequate absorbance (500–600 nm) to confirm sufficient cell abundance. Once confirmed, *E. coli* cultures were spun down into pellets, rinsed with 0.9% NaCl, washed in DI water and stored at -40 °C until δ^{13}C isotope analyses.

The δ^{13}C-labeled media (Dg-13C® Sigma-Aldrich, St. Louis, MO) used to culture experimental *Escherichia coli* cells were grown into pellets prior to the laboratory feeding experiments and subsequently measured for isotopic value differences as part of the overall study. An additional goal was to measure the isotopic fractionation value (*i.e.*, 1‰ for δ^{13}C; 3.4‰ for δ^{15}N) associated with primary source to consumer food web interactions. This procedure helped to evaluate trophic separation between microbial consumers and nutrient sources (Kreuzer-Martin *et al.* 2004). Microbial consumers can reflect isotopically labeled media (Kreuzer-Martin and Jarmen 2007). To test whether the strength (greater ‰ value) of label would have an effect on tissue isotopic signatures once ingested, two distinct recipes were developed, which included a weak and strong dose of the δ^{13}C label material.

Bivalve collection

Adult *C. fluminea* (mean length 20 ± 0.5 mm) and *E. complanata* (mean length 45 ± 2 mm) were collected at a university lake research site (35°41′37″N, 78°42′12″W) located in the Neuse River Basin, Raleigh. Water quality in this system was considered within the state standard limits (NCDENR 2009). The bivalve specimens were collected by snorkeling at water depths from 1.5 to 3 m; depending on lake level fluctuations. After separation of living bivalve specimens from debris, sand and gravel, samples were placed in flow-through systems with filtered (< 30 μm), aerated lake water at an ambient temperature of 20 °C until the start of feeding experiments. This initial holding period of 24 hours was used to depurate the stomach contents of each individual bivalve while adequately eliminating bacterial content from prior residence.

Experimental setup

Aquaria Set-up

Prior to the feeding experiments, 30 glass jars (2L) were filled with dechlorinated tap water and aerated for 7-days prior to each treatment. Thirty-three *E. complanata* were placed in 11 of the experimental glass jars (3 per jar) and 55 *C. fluminea* were placed in 11 jars (5 per jar). A daily light/dark regime of 16:8 h was maintained throughout the experiments. Conductivity, pH, dissolved oxygen, and temperature were measured daily using a YSI® Sonde in compliance with EPA quality control guidelines (EPA 1993). Water exchange (¼ of total jar volume) was performed every 2nd day.

Feeding procedure

Diet treatments included *E. coli* cells (labeled and unlabeled) grown from frozen stock cultures (Table 1). The stored frozen media was thawed and inoculated with stock *E. coli* cells, and incubated for 24 to 48 hours. To confirm adequate

Table 1. Labeled and unlabeled treatments describing each method. Note: e = *Elliptio*; c = *Corbicula*

Treatment ID	Type	Diet Source Treatment	Jar ID	Method
1	Unlabeled	100% algae (Nanno Brand A)	1-3e, 2-5c	Daily dose (mixture of algal cells in water) included 2 drops or 0.11 g by volume).
2	Unlabeled	100% algae (Shellfish Brand B)	3-3e, 4-5c	Same as 1A.
3	Labeled	100% δ^{13}C label pellet (Brand $A_{WEAK} = \delta^{13}C_6$ 0.08 g/1000 mL LB-B)	5-3e, 6-5c	Labeled mixture and live cultured cell cultures added daily (0.2 g = 2 mL) per jar.
4	Labeled	100% δ^{13}C label pellet (Brand $B_{STRONG} = {}^{13}C_6$ 0.16 g/1000 mL LB-B)	7-3e, 8-5c	Same as 3LpW.
5	Labeled	100% δ^{13}C label pellet (Brand $A_{WEAK} = {}^{13}C_6$ 0.08 g/1000 mL LB-B)	9-3e, 10-5c	Same as 3LpW.
6	Labeled	100% δ^{13}C label pellet (Brand $B_{STRONG} = {}^{13}C_6$ 0.16 g/1000 mL LB-B)	11-3e, 12-5c	Same as 3LpW.
7a	Labeled	Algae (Nanno Brand A)	13-3e, 14-5c	Algae Nanno mix (0.04 g $\delta^{13}C_6$ labeled dissolved in 5 mL of DI H_2O (1g = 1mL) with $\delta^{13}C_6$ label added 2 mL per day per jar.
7b	Unlabeled	1:1 Algae Nanno mix: Detritus 100% (unfiltered - leaf blended).	15-3e, 16-5c	2 mL drops were added per day per jar.
8	Labeled	Detritus	17-3e, 18-5c	1- Mixed 5 g (wet wt. 1:1 org matter to DI water) of detritus (unfiltered) with *E. coli* in 0.04g of $\delta^{13}C_6$ labeled mix dissolved in 5 mL of DI H_2O. 2- Added 0.2 g of per day.
9	Unlabeled	1:1 or 100% = Mix 5 g (wet wt.) detritus dissolved in 5 mL of DI H_2O.	19-3e, 20-5c	2 mL of detritus (filtered) added per day per jar.
10	Unlabeled	Same as 9DF.	21-3e, 22-5c	Same as 9DF except unfiltered.
11	Unlabeled	Water only	23-3e, 24-5c	Baseline treatment

cell growth, a Nanodrop® spectrophotometer was used to measure absorbance at the completion of the incubation stage. Each day of the experiment both labeled and unlabeled groups were removed from refrigeration and thawed for 10 minutes. Both groups were inoculated, incubated at 37 °C for 30 minutes, spun down (300 rpm/5 min) then the re-suspended pellet was gently agitated in a vortex for 10 seconds without lysing cells. Pellets were used to dose treatment jars with a 2 mL of cell mixture. A consistent volume of 100 mL of *E. coli* cultured pellet was used with each treatment dose (2mg). Once jars mixed for a period of 1 minute, an aliquot sample of 2 mL was removed for flow cytometric quantification of cell concentrations and to confirm particle ingestion. Prior to dissection the shell was rinsed with deionized water. Approximately 2 g of material made of the stomach, gill and foot tissue were extracted and stored in a freezer at -20 °C until lyophilized. Foot tissue was examined as a negative control, since the experimental time period was not long enough to show assimilation (Tieszen *et al.* 1983, Gustafson *et al.* 2007).

Flow cytometry analysis

Flow cytometry (method to discriminate different sized particles) (Davey and Winson 2003) was used to confirm particle retention by bivalves in each treatment jar that corresponded with diet cell particles. A modular flow cytometer/cell sorter (Dako Cytomation Inc.), College of Veterinary Medicine, Flow Cytometry Laboratory, North Carolina State University, was used to quantify cell concentrations within each experimental jar. The method uses a high-speed cell sorter coupled with a UV laser detector to measure a unique fluorochrome emission wavelength or signature for each sample aliquot examined.

Statistical Analyses

Prior to the feeding experiment, stomach contents and tissue samples were measured in triplicate for $\delta^{13}C$ values to serve as a baseline for labeled treatments of *E. coli* retention before and after the 7-day feeding experiment. Subsequent measurements were made at the end of the experimental period. To test whether a particular diet treatment was retained by experimental bivalves, analyses were conducted between $\delta^{13}C$-labeled and unlabeled treatments in stomach content (SC) and gill (G) and foot (F) tissue. An analysis of variance (ANOVA), controlled for treatment jar, was used to identify differences in retention among labeled and unlabeled media treatments. To account for differences between each species (*C. fluminea* and *E. complanata*) and to increase sample size per treatment, the results were grouped together by anatomic structure. A Tukey *post hoc* test was used to test for significant differences among each anatomical section.

RESULTS

Water chemistry

Daily water chemistry measurements were recorded throughout the experiment and mean values for the entire 7-days showed stable water quality levels. Water temperature averaged 20 °C (± 0.5) for all jars across the entire experiment and pH was stable. In all jars, conductivity increased from 50 to150 µs/cm from day 0 to 7. This trend was evident regardless of treatment jar and not unexpected since dissolved organic matter (*i.e.*, urine and food waste) accumulates with time and can increase conductivity levels.

Daily measurements of water clarity were made using flow cytometry, a particle detection technique that is based on high-pressure liquid chromatography (HPLC). Flow cytometric analyses indicated that experimental bivalves retained more than 80% of water column particles. Each dietary treatment had a distinct fluorescence signature, and showed decreased abundance in suspension, which provided some evidence of bacterial retention over the 7-day feeding event.

Labeled and unlabeled treatments

Prior to the feeding experiment, growth media with and without a $\delta^{13}C$ label were used to culture bacterial cells from stock (*E. coli* stock#25922). Cell growth media was filtered and results from a dual isotopic ($\delta^{15}N$ and $\delta^{13}C$) model showed that mean $\delta^{13}C$ values of the unlabeled media (*i.e.*, M9, LBB, and TRB) were significantly more negative than the labeled samples ($p < 0.01$).

From the filtered media, a series of isotopic experiments were conducted with bacterial cultures to be used as treatments with and without a $\delta^{13}C$ label. Pelleted *E. coli* cultures reflected an observable trend that showed unlabeled samples to be substantially less in $\delta^{13}C$ value compared to labeled samples. Both labeled groups were at least 70‰ greater in $\delta^{13}C$ than unlabeled groups, which provided a reliable distinction to use as dietary treatments.

Feeding study

There were significant differences in isotopic values among labeled versus unlabeled anatomic tissues for both bivalve species tested. The overall ANOVA for the 7-day experiment controlling for all three anatomic structures (*i.e.*, stomach contents, gill, and foot) showed a significant difference in $\delta^{13}C$ values among treatments (24 jars, $df = 140$; $F = 4.88$; $p < 0.001$). To further evaluate evidence of bacterial particle retention in the stomach cavity for bivalve species, a Tukey HSD *post-hoc* comparison test was performed controlling for stomach contents (Table 1). A significant difference was apparent between 100% $\delta^{13}C$ labeled treatment 4 (Brand $B_{STRONG} = {}^{13}C_6$ 0.16 g/1000 mL LB-B) and unlabeled 1:1 algae Nanno mix treatment 7b ($df = 54$; $F = 4.9$; $P < 0.05$) (Fig. 1). Among other types of treatment combinations, differences were observed with labeled and unlabeled groups, although not statistically significant likely due to sample size. For example, there was a greater than 20‰ $\delta^{13}C$ difference or fractionation between 100% $\delta^{13}C$ labeled treatment diet 5 (Brand $A_{WEAK} = {}^{13}C_6$ 0.08 g/1000 mL LB-B) and unlabeled detritus treatment 9. In addition, 100% $\delta^{13}C$ labeled treatment 6 (Brand $B_{STRONG} = {}^{13}C_6$ 0.16 g/1000 mL LB-B) compared to unlabeled algae Nanno mix treatment 7b and unlabeled treatment 11 showed greater than a 10‰ fractionation. The magnitude of fraction difference observed in this study is considered meaningful in terms of food source interactions (Peterson and Fry 1987, Michener and Schell 1994, McClelland and Valiela 1998, Kendall *et al.* 2001, Anderson and Cabana 2005).

Results of *C. fluminea* and *E. complanata* gill tissue also showed a significant difference in $\delta^{13}C$ values ($F = 13.57$; $df = 31$; $P < 0.0001$) among a wider range of labeled and unlabeled treatments compared to stomach contents (Fig. 2). Significant differences were observed among the following combinations; 3 and 7b, 8, 9, 5; 4 and 7, 8, 9, 5; 6 and 7b, 8, 9; 5 and 7b, 8, 9, 5. Labeled detritus and *E. coli* (8, 9) as a diet was significantly different compared to *E. coli* (only) treatments (*e.g.*, 3, 4, 5; 6). Foot tissue did not show significant differences among all treatments for both *Elliptio* and *Corbicula* tested (Fig. 3). For all tissues examined, variability between the strength of the $\delta^{13}C$ label (weak vs. strong) showed no significant difference among diet treatments for either bivalve species.

DISCUSSION

We have a limited understanding of the role that environmental bacteria play in the nutritional health of freshwater

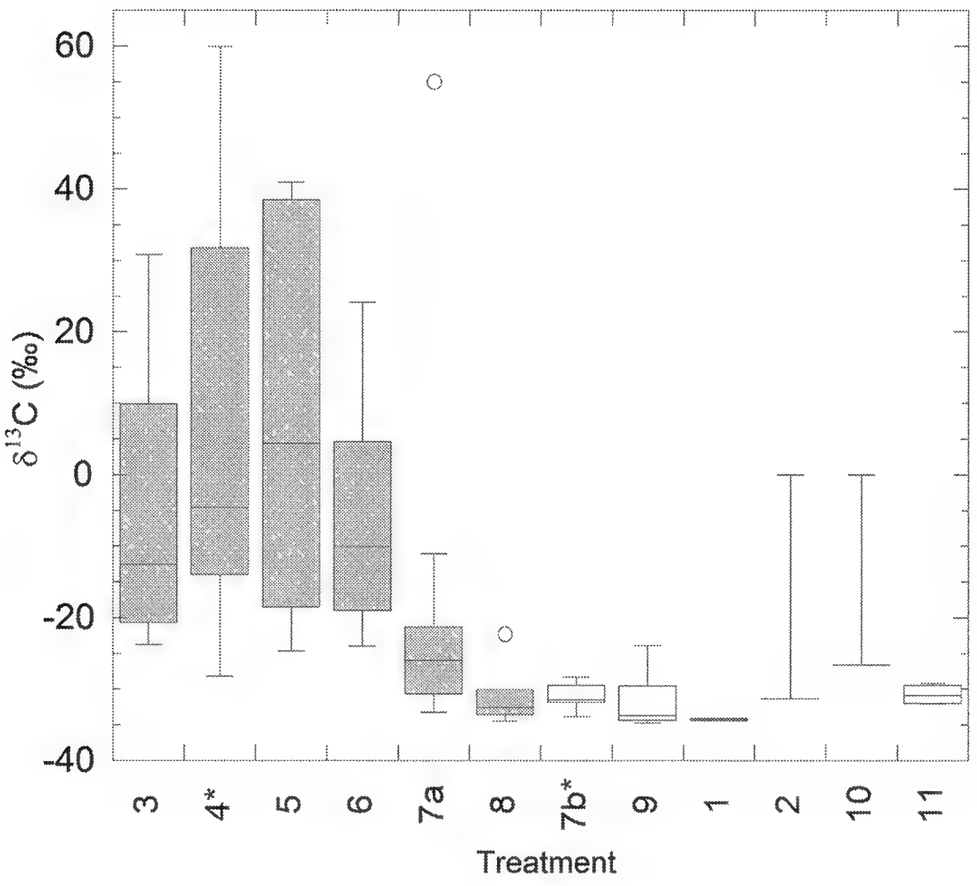

Figure 1. The δ^{13}C values of stomach contents for labeled (gray) and unlabeled (white) treatments. Starred treatments were significantly different at the $P < 0.05$ level. The boxes represent the 25–75% range and whiskers are 10% and 90%. The line within each box represents a median value, which means that 50% of the ranked values are above and 50% below the line. The outliers are points that are outside the 10–90% range.

bivalves. Prior studies using cultured microorganisms suggest that these bivalves have the ability to remove bacteria from suspension (Riisgård *et al.* 2003). However, there is little consensus about whether or not bacteria such as *E. coli* are retained and thus accumulated as gastrointestinal flora (Kueh and Chan 1985). Studies have shown significant differences in the particle size, quantity and generic composition of bacterial biota ingested from seawater by the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) and the clam, *Scapharca cornea* (Reeve, 1844) (Cognie *et al.* 2001, Kang *et al.* 2009). For example, *Pseudomonas* spp. was the dominant bacteria with *Vibrio* spp., *Acinetobacter* spp., and *Aeromona* spp. also isolated. In one study, *Aeromonas hydrophlia*, other *Enterobacter* spp. and *Bacillus* spp. were the most prevalent bacteria isolated from *E. complanata* collected in North Carolina streams (Chittick *et al.* 2001). Our research supports the hypothesis that water column *E. coli* are retained on the gill tissue of both *E. complanata* and *C. fluminea* and subsequently transferred to the stomach (Figs. 1 and 2). Perhaps *E. coli* cells aggregate on the gill tissue during sorting and assimilation processes, which may serve to entrain particles prior to being ingested by the stomach.

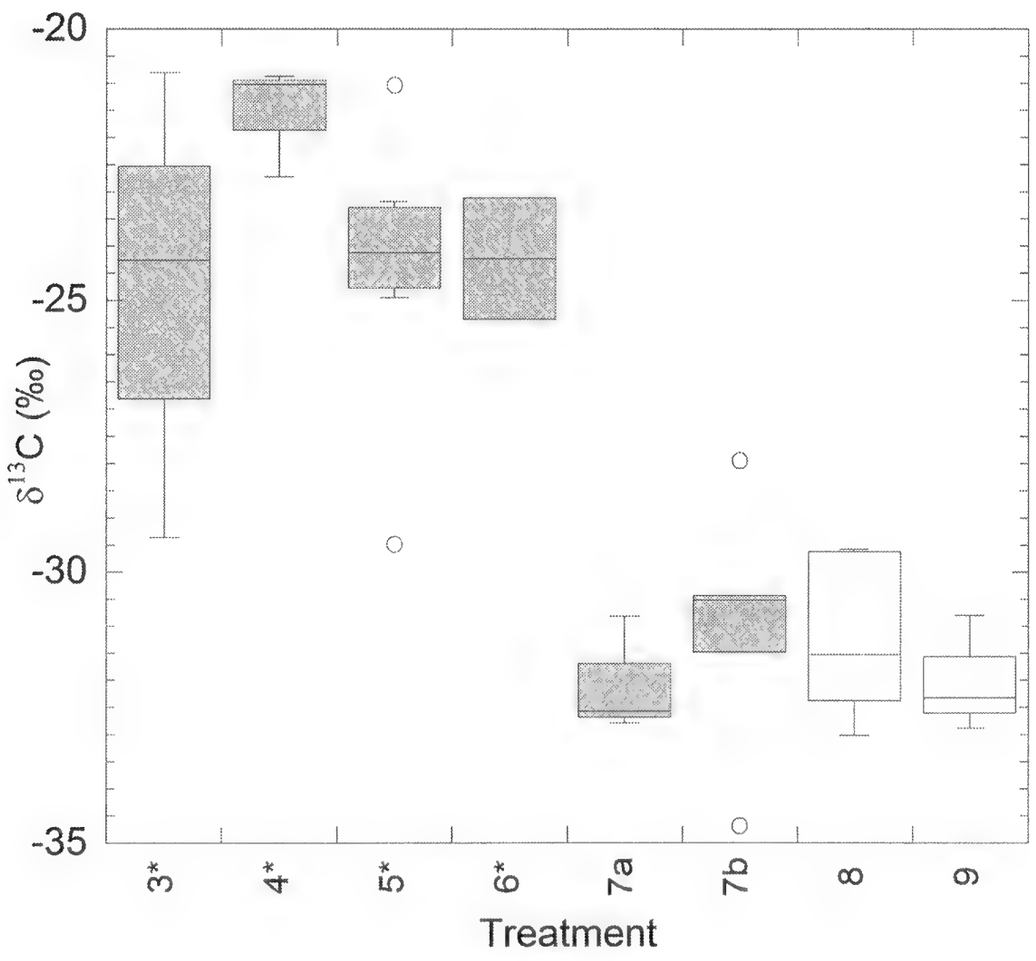

Figure 2. Gill tissue analyzed with and without δ^{13}C label across treatments. (* = significant; $P < 0.05$).

Environmental bacteria as a dietary source

Similar to marine species, adult freshwater bivalves clear and sort suspended organic matter particles ranging in size from 0.9 to 250 μm (Kryger and Riisgård 1988, Silverman *et al.* 1997, Atkinson *et al.* 2010). This size range included bacteria, algal cells, and fine particulate detritus. Some aquatic bacteria available to filter feeding bivalves are less than 1 μm in diameter, a small size that may be efficiently retained on the gill of most bivalves when accumulated (Riisgård 1988, Dame 2011). Particle contact is likely facilitated by inertial impaction, gravitational deposition, diffusion or direct interception (Riisgård and Larsen 2010). Retention of the encountered particles is facilitated by gill cilia that serve as sieving structures (Jørgensen *et al.* 1984). Water velocity, particle size and shape, species specific differences in cilia shape and orientation further affect the retention and eventual transfer of the particles to the digestive tract for potential assimilation (Silverman *et al.* 1999, Riisgård and Larsen 2010).

Evidence of bivalve bacteria accumulation

Stable isotope studies that investigate tissue, including muscle, have demonstrated that there is marked variability in the food resources used by different bivalve species in streams (Wen *et al.* 2009, Atkinson *et al.* 2010). *Elliptio complanata* filters fecal coliform bacteria (*i.e.*, *Escherichia coli*) from a variety of freshwater stream environments (Gewurtz *et al.* 2011). Studies using radiolabelling (^{14}C) techniques have shown that *E. coli* can be utilized as a preferred food source when fed to the marine bivalve *Mytilus edulis* (Linnaeus, 1758) (McHenery and Birkbeck 1985, Lauritsen 1986). *Escherichia coli* were accumulated in the digestive gland, mantle, gill, and body of the *M. edulis* based on filtration rate. A subsequent study suggested that *M. edulis* digestion of ^{14}C-labeled bacteria appeared to be initiated extracellularly in the stomach and completed intracellularly in the digestive

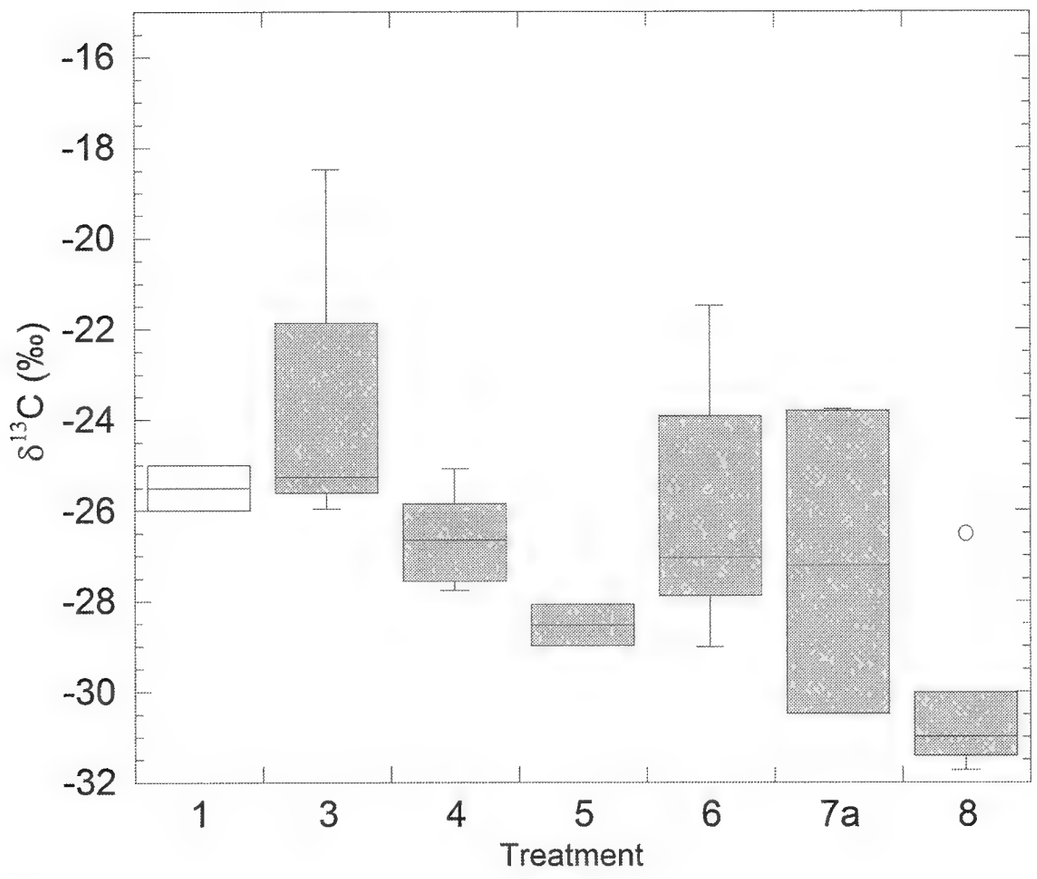

Figure 3. Foot tissue analyzed with $\delta^{13}C$ label across a range of treatments.

gland (Turick *et al.* 1988). Studies by Nichols and Garling (2000), and Christian (2004) using C^{13} stable isotope analysis documented the role bacteria may play as integral components of freshwater mussel diets. Although stable isotope studies have informed potential assimilation processes, stable isotope data in dietary studies can also be effective as tracers of various food items. Strayer (2008) and others have suggested that individual anatomical structures be evaluated. Our study documented differences in retention, which is relevant to digestion, nutrition and ultimately survival.

The results of this study are consistent with the knowledge that gills are anatomical structures that actively contribute to the retention of bacterial particles (Baker *et al.* 1998, Dyhrman *et al.* 2010). *Escherichia coli* cells may likely be retained on gill tissue and to a lesser degree in the stomach of *C. fluminea* and *E. complanata*. Freshwater bivalves may compensate for reductions in food quality, and thereby maintain energy intake by altering the retention rate and sorting efficiency (Silverman *et al.* 1995, Nichols *et al.* 2005, Bucci *et al.* 2008).

In aquatic systems that are being degraded by land-use activities, consequent changes in the composition of water column food and sediments available to mussels may be markedly altering microbial communities (Fierer *et al.* 2007, Hutchins 2010). These isotope tracer studies examining the retention of *E. coli* on the gills and in the stomach of *C. fluminea* and *E. complanata* will support further investigation of the role of bacteria in the diet and the nutritional health of freshwater bivalves. Competitive inhibition of bacterial retention and ingestion by bacteria that are of little nutritional value could potentially be limiting nutrient assimilation by native freshwater mussels. Additional studies are needed to examine competitive inhibitive retention of bacteria, associated nutrient assimilation and the overall contribution of bacteria to freshwater mussel health.

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Nystiellidae (Gastropoda: Epitonioidea) collected during the REVIZEE Program/northeast Brazil with descriptions of new species and a checklist of the family from the Atlantic coast of South America

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Abstract: Four nystiellids belonging to the genera *Eccliseogyra* Dall, 1892, *Opaliopsis* Thiele, 1928 and *Papuliscala* de Boury, 1911 were collected on the continental slope off Brazil during the development of the REVIZEE Program (2000–2001). Of these *Opaliopsis atlantis* (Clench and Turner, 1952) was the only species known previously. Three nystiellids unknown to science, belonging to the genera *Eccliseogyra* and *Papuliscala*, are presented herein based on shell morphology. *Eccliseogyra maracatu* sp. nov. and *Papuliscala nordestina* sp. nov. are described for northeastern Brazil. Only one specimen of *Eccliseogyra* sp. with damaged shell was found. A formal epithet for this specimen will be delayed until additional material is collected. A checklist of species of Nystiellidae Clench and Turner, 1952 known for the Atlantic coast of South America, as well as their geographic and bathymetric distribution based on data from the literature, is presented.

Key words: biodiversity, *Eccliseogyra*, *Opaliopsis*, *Papuliscala*, deep water.

Micromollusks comprise a large proportion of unknown metazoans in oceans (Bouchet *et al.* 2002) and also constitute an enormous contingent of species threatened with extinction (Haszprunar *et al.* 2008).

Most members of the family Nystiellidae Clench and Turner, 1952 are deep-sea microgastropods that are rarely collected in marine regions worldwide (Watson 1886, Bouchet and Warén 1986, Weil *et al.* 1999). Known occurrences are usually associated with coral communities (Beu and Climo 1974, Beu 1978, Kilburn 1985, Bouchet and Warén 1986). The current degradation of coral communities (Sandin *et al.* 2008) certainly threatens the existence of Nystiellidae and other families of microgastropods that feed mainly or exclusively on cnidarians (Lima *et al.* 2012).

The first nystiellids were discovered in the nineteenth century during pioneering oceanographic prospecting of the deep-sea in the Atlantic Ocean (Jeffreys 1877, Watson 1886, Verrill 1885, Dautzenberg and Fischer 1896, Dautzenberg and de Boury 1897). A number of open-sea oceanographic expeditions that followed have expanded our knowledge on the species richness of benthic communities around the world (Costello *et al.* 2010, Miloslavich *et al.* 2011), including nystiellids (Sykes 1925, Dall 1927, Clench and Turner 1952, Bouchet and Warén 1986, Castellanos *et al.* 1987, Sabelli and Taviani 1997). Towards the end of the twentieth century, Weil *et al.* (1999) summarized the knowledge on recent nystiellids. Sporadic records of the group have since been published for restricted regions, mainly on the Atlantic coast of South America (Forcelli 2000, Miyaji 2004, Benkendorfer and Soares-Gomes 2009, Rios 2009, Andrade *et al.* 2011, García 2011).

This paper presents three nystiellids previously unknown to science, referable to the genera *Eccliseogyra* Dall, 1892 and *Papuliscala* de Boury, 1911, dredged from the continental slope off northeastern Brazil during the REVIZEE (Live Resources of the Exclusive Economic Zone) Program (2000–2001). In addition, a checklist is presented summarizing current knowledge on the biodiversity of Nystiellidae from the Atlantic coast of South America.

MATERIALS AND METHODS

The checklist presented here is based primarily on data from the literature. Table 1 summarizes the information on species richness, and the geographic and bathymetric distribution of Nystiellidae along the Atlantic coast of South America.

Generic identification and specific comparisons of the taxa dredged from deep waters off Brazil are based on Bouchet and Warén (1986), Weil *et al.* (1999) and García (2011). The count of the whorls (accuracy of 1/8) on the protoconch and teleoconch is based on Bouchet and Kantor (2004: 468). Each species was photographed under a ZEISS EVO 40 scanning electron microscope at the "Gerência de Bioestratigrafia e Paleoecologia Aplicada (BPA)" of the "Petrobrás Research Center (Centro de Pesquisas da Petrobrás – CENPES)", Rio de Janeiro, Brazil.

Abbreviations

IBUFRJ Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil;

Table 1. Checklist of Nystiellidae known for the Atlantic coast of South America (SA) with geographic and bathymetric distribution.

Species	**Distribution** (SA)	**Depth (m)**	**References**
Eccliseogyra brasiliensis García, 2011	Brazil: Espírito Santo to Rio de Janeiro.	610–1550	García (2011)
Eccliseogyra nitida (Verrill and Smith, 1885)	Brazil: Pernambuco.	640–645	Watson (1886), Clench and Turner (1952), Rex and Boss (1973), Bouchet and Warén (1986)
Eccliseogyra cf. *sericea* Bouchet and Warén, 1986	Southeastern Brazil	Depth not given	Miyaji (2004)
Eccliseogyra sp.	Brazil: Pernambuco	690	Present study
Eccliseogyra maracatu sp. nov.	Brazil: Alagoas	720	Present study
Narrimania azelotes (Dall, 1927)	Southeastern Brazil	Depth not given	Miyaji (2004)
Narrimania cf. *concinna* (Sykes, 1925)	Southeastern Brazil	Depth not given	Miyaji (2004)
Opaliopsis atlantis (Clench and Turner, 1952)	Brazil: Ceará, Pernambuco, Rio de Janeiro, Paraná, Santa Catarina	150–690	Rios (2009), Andrade *et al.* (2011)
Opaliopsis cearense Andrade, Costa and Pimenta, 2011	Brazil: Ceará	240–260	Andrade *et al.* (2011)
Opaliopsis opalina (Dall, 1927)	Brazil: Ceará to Rio de Janeiro	240–260	Andrade *et al.* (2011)
Opaliopsis cf. *atlantis* (Clench and Turner, 1952)	Southeastern Brazil	Depth not given	Miyaji (2004)
Opaliopsis sp.	Southeastern Brazil	Depth not given	Miyaji (2004)
Papuliscala diminuta Castellanos, Rolán and Bartolotta, 1987	Argentina	600	Castellanos *et al.* (1987)
Papuliscala nordestina sp. nov.	Brazil: Alagoas	720	Present study

MNRJ Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil;
REVIZEE Live Resources of the Brazilian Exclusive Economic Zone;

RESULTS

Taxonomy

Epitonioidea Berry, 1910
Nystiellidae Clench and Turner, 1952
Eccliseogyra Dall, 1892

Genus characterization

Shell thin, fragile, whitish, small (about 3.1 mm in length) to relatively large, slender (reaching about 26 mm in length), trochiform to turriculate. Protoconch conical, multispiral, with 2.5 to 4 whorls attached, evenly rounded; suture incisive, moderately deep. Apical nucleus usually smooth and remaining sculptured with numerous, rather sigmoid, strong axial riblets and rather raised, fine spiral threads between riblets. Teleoconch with 3.5 to 12 whorls rather loosely coiled, freely coiled or with coiling variable; rather constricted, rounded, evenly convex or with peripheral angulation slightly above the middle possibly forming shoulder. Spire short to extended. Suture indistinct (teleoconch freely coiled) or incisive, rather deeply impressed (teleoconch loosely coiled). Axial sculpture consisting of blade, thread-like ribs or lamellae numerous, regularly spaced, fragile, fine (sometimes crisp, serrated), usually prosocline, somewhat sigmoid, low to moderately high; ribs or lamellae crossing or not over suture, without spines or projecting spines usually at periphery that may be reflected backwards. Spiral sculpture consists of numerous, fine, attenuate, rounded, low or strong, raised, rounded threads, rather evenly arranged over the surface of each whorl, usually not crossing over axial ribs/lamellae. Basal disc indistinct or well delineated by a ridge usually ornamented with obsolete or well-delineated ribs/lamellae and threads. Umbilicus indistinct; small, very narrow, narrowly open or widely open. Umbilical region usually with ribs/lamellae axial and spiral threads more closely set. Aperture slightly oval to subcircular, may be holostomatous and/or angular towards the base in some species. Outer lip thin. Inner lip usually thin, may expand slightly over umbilical area or reflected over columella.

Eccliseogyra maracatu sp. nov. (Figs. 1A–E)

Description

Shell white, thin, conical, 4.2 mm length (Fig. 1A). Protoconch brownish, conical with 4¼ rounded whorls: 1¼ whorls smooth, remaining whorls sculptured with axial ribs and weak

slight spiral threads (Figs. 1B–C). Teleoconch with about 3½ rapidly increasing whorls; whorls loosely coiled, strongly convex, inflated, rounded (Fig. 1A). First post-nuclear whorl much larger than the protoconch (Figs. 1A–B). Suture deep and constricted (Fig. 1A–B, E). Axial sculpture of faint, sigmoid, prosocline threads (Figs. 1A, E). Spiral sculpture of rounded threads, slightly stronger than axial sculpture (Figs. 1A, E). Penultimate whorl with about 16 spiral and 36 axial threads (Fig. 1A). Last whorl with 28 spiral threads and 36 axial threads (Fig. 1A); spiral and axial threads forming rectangular interspaces (Fig. 1A). Base rounded, concave around umbilicus (Figs. 1A, D). Umbilicus deep, rather large (Fig. 1D). Aperture ample, subcircular (Fig. 1D); outer lip thin (Fig. 1D); inner lip expanding over umbilical area (Fig. 1D).

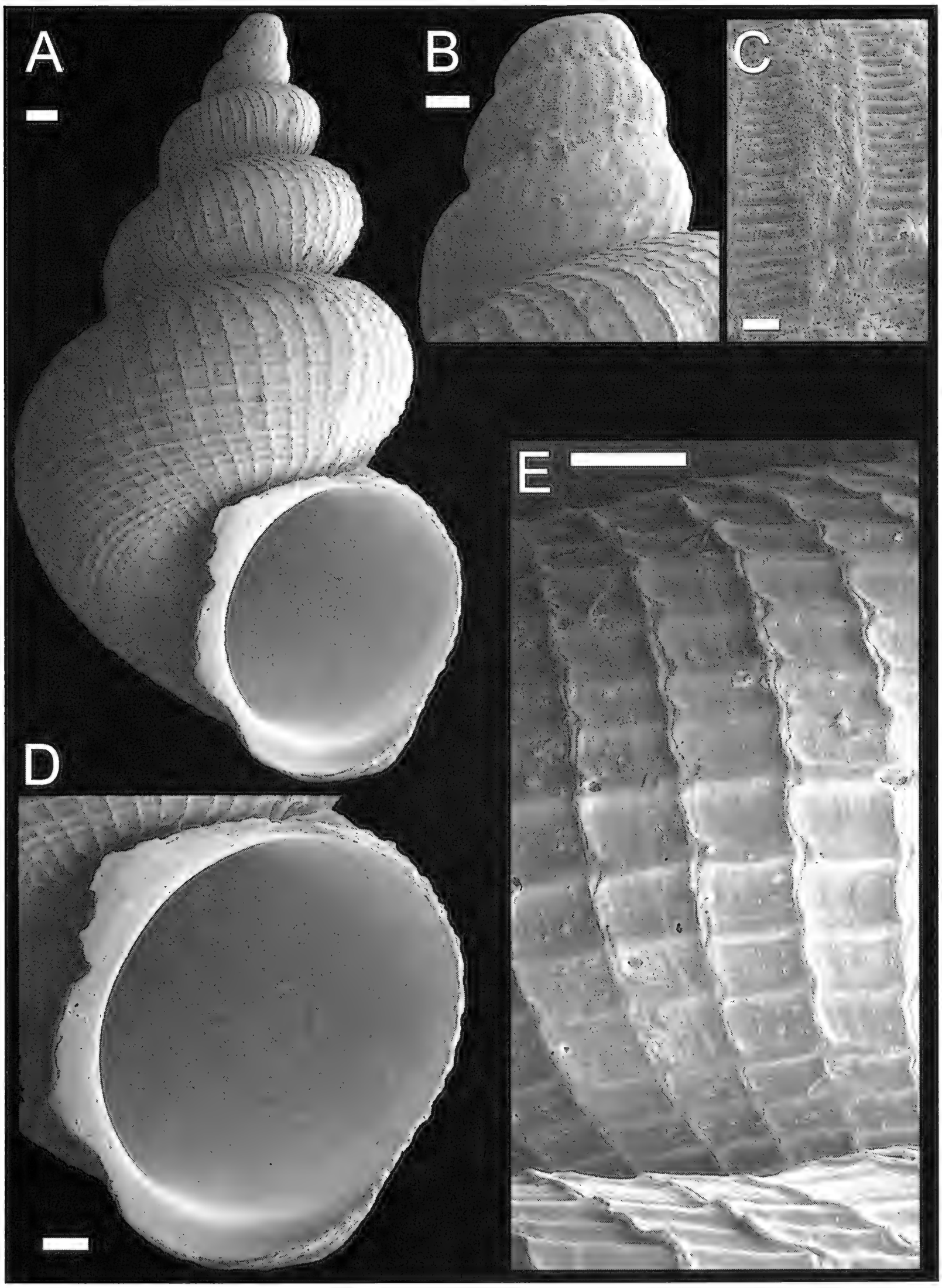

Figure 1A–E. *Eccliseogyra maracatu* sp. nov. (holotype, IBUFRJ 18.825): **A,** ventral view, **B,** protoconch, **C,** detail of protoconch ornamentation, **D,** apertural view, **E,** detail of teleoconch ornamentation. Scale bars: A: 500 μm, B: and E: 100 μm; C: 10 μm: and D: 200 μm.

Etymology

"Maracatu" is a cultural manifestation of African-Brazilian origin now expressed in folk music in northeastern Brazil.

Geographic distribution

Known only from the continental slope of Alagoas (northeastern Brazil).

Holotype

IBUFRJ 18.825 - Alagoas (Brazil - REVIZEE/NE: R/V Natureza, 10°06′35″S, 35°46′41″W, 720 m, 16.xii.2001).

Paratype

IBUFRJ 19.231 (juvenile) - Alagoas (Brazil - REVIZEE/NE: R/V Natureza, 10°06′35″S, 35°46′41″W, 720 m, 16.xii. 2001).

Type locality

State of Alagoas, 10°06′35″S, 35°46′41″W, at a depth of 720 m.

Remarks

Given that a number of nystiellids have shown amphi-Atlantic distribution (Rex and Boss 1973, Bouchet and Warén 1986, Weil *et al.* 1999), eastern Atlantic congeners also need to be compared with the species described herein.

The shell morphology of *Eccliseogyra maracatu* sp. nov. differs considerably from Atlantic congeners. There are only two species of *Eccliseogyra* previously reported for Brazil: *E. nitida* (Verrill and Smith, 1885) and *E. brasiliensis* García, 2011.

Eccliseogyra maracatu sp. nov. and *E. nitida* share about 3½ whorls on the teleoconch, prosocline and sinuous axial threads, rounded spiral threads and about four whorls on the protoconch. *Eccliseo-*

gyra maracatu sp. nov. differs from *E. nitida* by presenting inflated, rounded and loosely-coiled whorls, a deep and constricted suture, about 28 spiral and 36 axial threads on the last whorl and an inner lip reflected over the umbilical area. *Eccliseogyra nitida* has open coiling, about 17 spiral threads and 27 axial ribs on the last whorl (Watson 1886: 142, pl. 9, fig. 6, Clench and Turner 1952: 347, pl. 170, fig. 1–2, Bouchet and Warén 1986: 482, fig. 1139, Rios 2009: 186, fig. 455).

Eccliseogyra maracatu sp. nov. and *E. brasiliensis* are similar only in presenting a thin shell, sigmoid ribs, subcircular aperture and inner lip reflected over the umbilical area. *Eccliseogyra maracatu* sp. nov. differs from *E. brasiliensis* by having a conical shell, about 3½ whorls on the teleoconch, whorls not shouldered at the periphery, a deep, constricted suture, axial and spiral threads, no basal disk and a deep, rather large umbilicus. *Eccliseogyra brasiliensis* has a rather widely turriculate shell, six whorls on the teleoconch that develop a roundly angular shoulder at the periphery, an incised suture, axial lamellae, stronger spiral elements, a prominent basal disk and a very narrow umbilicus (García 2011: 167–169, figs. 1–4).

Eccliseogyra maracatu sp. nov. is somewhat similar to *E. monnioti* Bouchet and Warén, 1986 [abyssal zone of the eastern Atlantic] in its fragile shell, whorls loosely coiled and strongly convex, inflated and rounded, the number of axial and spiral threads on the last and penultimate whorl, well-impressed, deep, constricted suture and numerous undulating axial sculpture evenly distributed over the surface of the whorls, differing by the presence of a conical shape, whorls not free from each other, weak spiral threads and aperture not angular towards the base. *Eccliseogyra monnioti* has a trochiform shell, highly variable coiling, strong spiral threads and aperture somewhat angular at the base (Bouchet and Warén 1986: 483–484, figs. 1140–1141, type material; Weil *et al.* 1999: 36, fig. 87).

Eccliseogyra sp. (Figs. 2A–C)

Geographic distribution

Known only from the continental slope of Pernambuco (northeastern Brazil).

Material examined

IBUFRJ 18.826 (1 shell) - Pernambuco (Brazil - REVIZEE/NE: R/V Natureza, 08°46′00″S, 34°44′00″W, 690 m, 18. xi.2000).

Remarks

Despite the damaged shell, this species exhibits the distinguishing characters of *Eccliseogyra*. The teleoconch whorls have strong axial lamellae and spiral cords in a pattern similar to that found on *E. brasiliensis*, *E. exquisita* Bouchet and Warén, 1986, *E. folini* (Dautzenberg and de Boury, 1897) and *E. formosissima* (Jeffreys, 1884).

Eccliseogyra sp. is similar to *E. brasiliensis*, *E. exquisita*, *E. folini* and *E. formosissima* only in the conical shape and strength and number of axial lamellae and spiral cords on the teleoconch, but it is distinguished by the absence of an angular shoulder at the periphery.

Thus, we suspect that *Eccliseogyra* sp. is a new species to science, but a formal epithet will be delayed until better material is available.

Papuliscala de Boury, 1911

Genus characterization

Shell solid, whitish, small (about 3.6 mm in length) to slender, much higher than broad (reaching about 7.6 mm in length), conical to turriculate. Protoconch globular, bulbous, rounded, inflated, paucispiral, with a little less than 1.5 to 2 convex whorls, usually with a smooth apical nucleus and remaining sculptured with low, faint, rather thick, orthocline to opisthocline axial riblets; suture incisive. Teleoconch with 5 to about 11 whorls, rather slightly to moderately constricted, rounded, regularly convex or with slight to well-developed peripheral angulation above the middle whorl. Spire rather extended. Suture incisive, moderately to deeply impressed. Axial sculpture consisting of prosocline, slightly sigmoid in some species, non-lamellar, rounded, strong to very strong, broad, rounded, thick ribs. Spiral sculpture consisting of obsolete, slight to well-developed (2 to 6 per whorl), strong, rounded threads or very strong, broad, thick, rounded ribs. Axial sculpture stronger than spiral or axial and spiral sculpture with equal strength forming cancellate pattern. Intersection of sculpture forming small to strong, high, rounded nodules with interspaces sub-square to square, shallow to deep. Intersection of sculpture may present spine at periphery of whorl. First teleoconch whorls and subsutural region may present indistinct spiral sculpture. Basal disc well-defined, very distinct, flat, slightly concave or convex, smooth or sculptured only with incremental threads or incremental threads and obsolete spiral ribs. No umbilicus. Aperture oval to rounded, may be slightly angular at base. Outer lip rather thin to thick.

***Papuliscala nordestina* sp. nov.** (Figs. 3A–C)

Description

Shell white, solid, turriform, elongate, slender, 7.9 mm length (Fig. 3A). Protoconch and early postlarval whorls missing. Teleoconch with at least eight convex, strongly shouldered whorls sculptured with strong, prosocline, widely spaced axial ribs and slight spiral threads (Figs. 3A–C). Intersection of sculptures forming very weak nodules (Figs. 3A–C). Last whorl sculptured with five to six spiral threads (Fig. 3B). Penultimate whorl sculptured with four to five spiral threads (Fig. 3A). Other teleoconch whorls show three to four spiral

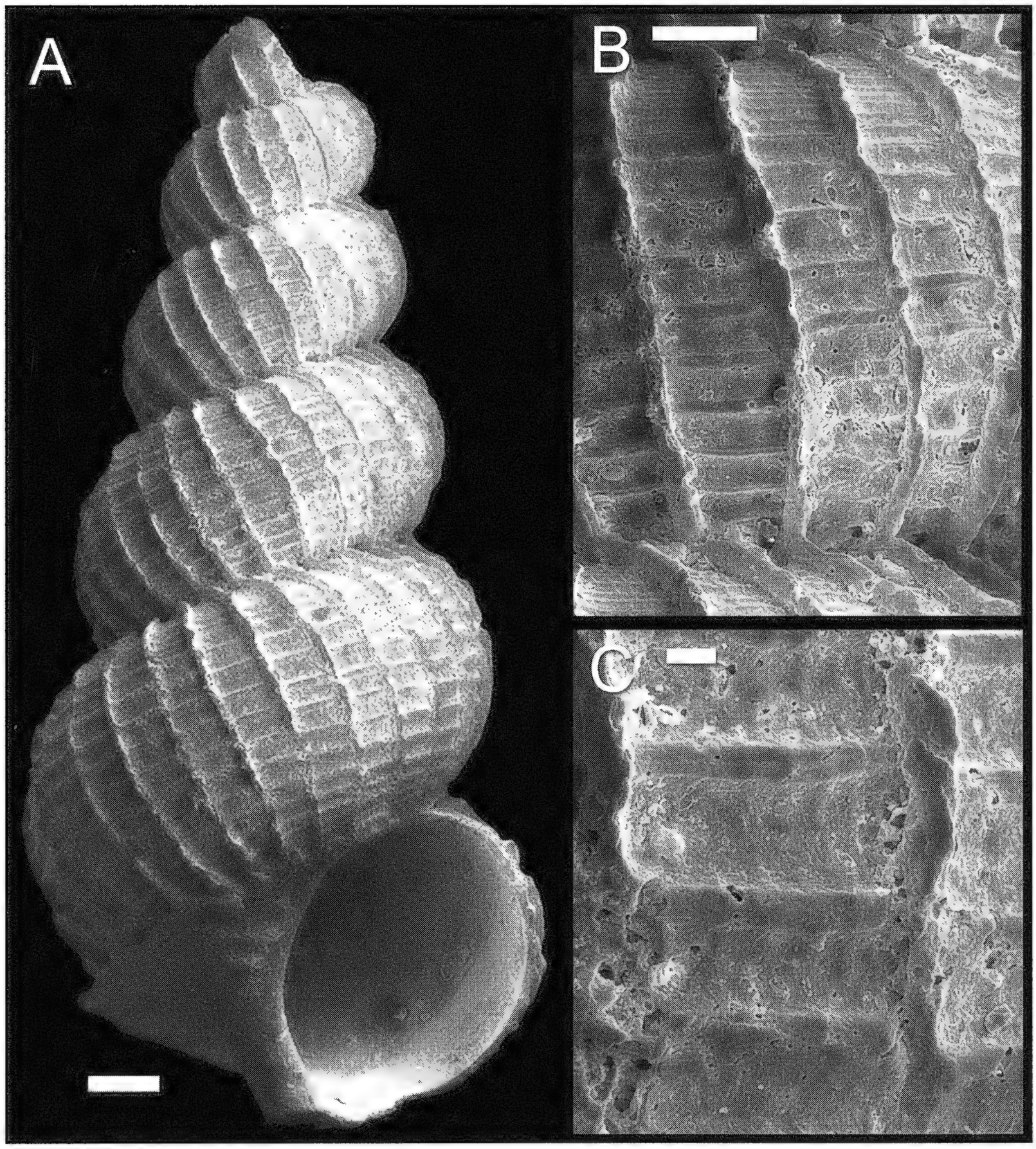

Figure 2A–C. *Eccliseogyra* sp. (IBUFRJ 18.826): **A**, ventral view, **B–C**, detail of teleoconch ornamentation. Scale bars: A: 500 μm, B: 200 μm, and C: 100 μm.

threads (Figs. 3A, C). Last and penultimate whorls sculptured with 13 to 14 axial ribs (Fig. 3A–B). Suture shallow (Figs. 3A, C). Sub-sutural area almost flat, shouldered, forming a prominent angulation on whorls (Figs. 3A, C), usually sculptured by axial ribs only (Fig. 3C). Aperture rounded (Fig. 3B). Umbilicus lacking (Fig. 3B).

Etymology

"Nordestino" is the common epithet given to natives of northeastern Brazil.

Geographic distribution

Known only from the continental slope of Alagoas, northeastern Brazil.

Holotype

IBUFRJ 18.827 - Alagoas (Brazil - REVIZEE/NE: R/V Natureza, 10°06′35″S, 35°46′41″W, 720 m, 16.xii.2001).

Type locality

State of Alagoas, 10°06′35″S, 35°46′41″W, at a depth of 720 m.

Remarks

Despite the damaged apex and corroded early teleoconch whorls, this species clearly exhibits the distinguishing characters of *Papuliscala*. Given the rarity of the group, it is characterized here based on a single specimen, with the hope that perhaps other specimens are housed elsewhere. The genus *Papuliscala* has previously been reported on the Atlantic coast of South America only from off Argentina (Castellanos *et al.* 1987).

Papuliscala nordestina sp. nov. and the Argentine species *P. diminuta* Castellanos, Rolán and Bartolotta, 1987 have in common only the strong axial ribs, rounded aperture and concave outer lip. The former differs by its turriform shell, with at least eight whorls on the teleoconch, shallow suture and whorls with peripheral angulation. In contrast, *P. diminuta* has a conical shell, five teleoconch whorls and a deep suture; it also lacks a peripheral angulation (Castellanos *et al.* 1987: 95, pl. 1 and 2, fig. 7).

Papuliscala nordestina sp. nov. is similar to *P. praelonga* (Jeffreys, 1877) in the turriform shape, subsutural angulation, prosocline, strong axial ribs, low, sparse spiral threads, with five spiral threads on the last and penultimate whorls (four spiral threads on other whorls), shallow suture and intersection of sculptures forming slight nodules. *Papuliscala nordestina* sp. nov. differs from *P. praelonga* by the presence of 13 to 14 axial ribs on the last and penultimate whorls, wider axial interspaces and a rounded aperture. In contrast, *Papuliscala praelonga* has at least 20 axial ribs on the last and penultimate whorls, narrower axial interspaces, less pronounced shoulder and an oval aperture (Bouchet and Warén 1986: 496, fig. 1162).

Papuliscala nordestina sp. nov. is strongly correlated with *P. elongata* (Watson, 1881) [eastern Atlantic: bathyal zone off southwest Europe and the Azores] in the turriform, elongate shell, elliptical contour of the whorls, subsutural region flattened developing a prominent angulation, suture slightly marked, about 13 axial ribs evenly spaced on the last whorl, three spiral threads on the most teleoconch whorls and intersection of sculptures forming nodules, differing by

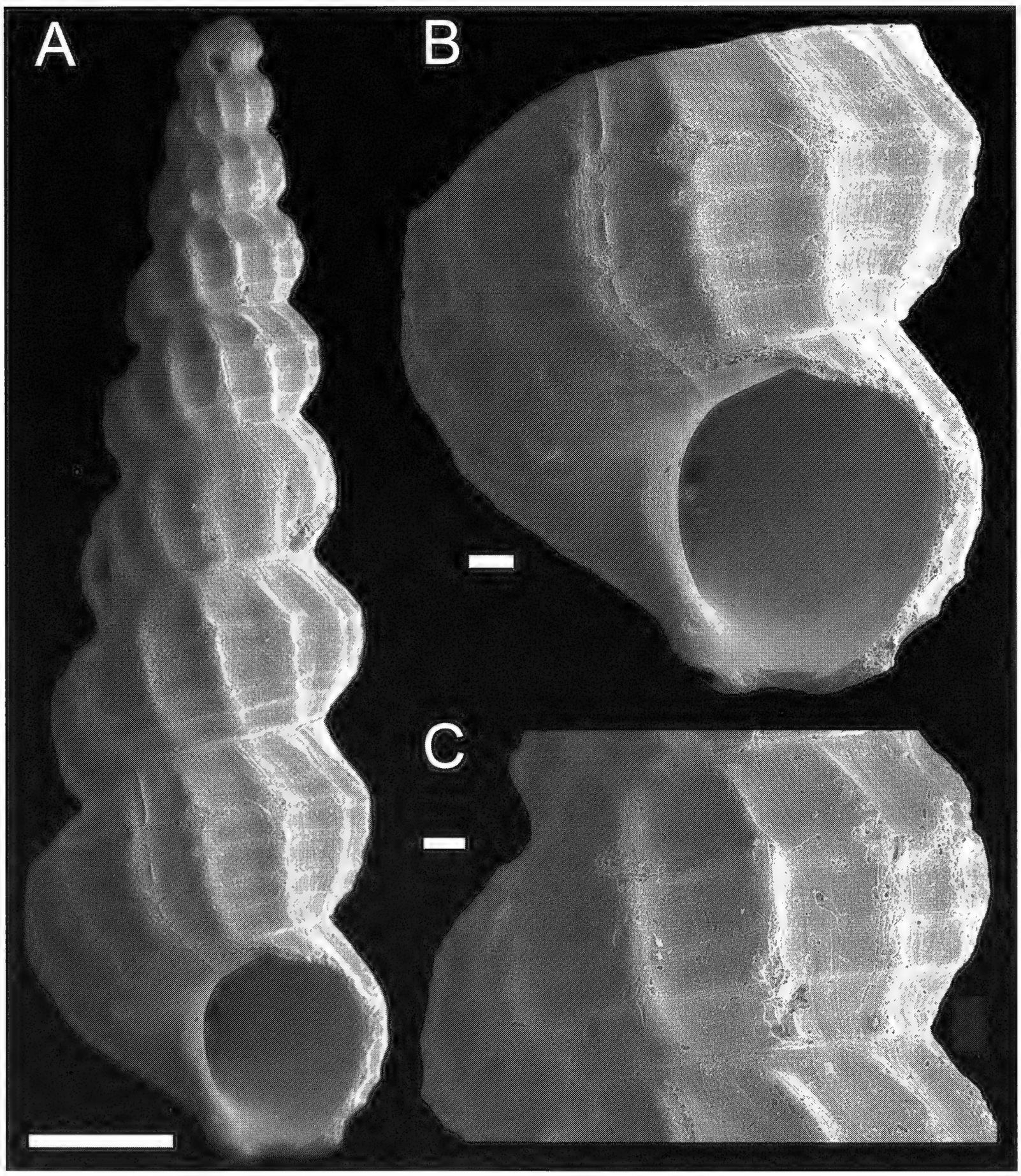

Figure 3A–C. *Papuliscala nordestina* sp. nov. (IBUFRJ 18.827): **A,** ventral view, **B,** view of body whorl, **C,** detail of teleoconch ornamentation. Scale bars: A–C: 500 μm.

the rounded aperture, non-spiny periphery of the whorls, subsutural region more widely flattened on penultimate and last whorl, and four to five and five to six spiral threads on the penultimate and last whorl, respectively. *Papuliscala elongata* develops an oval aperture, spiny periphery of the whorls, subsutural region narrowly flattened and three spiral threads per whorl (Watson 1881: 249; 1886: 621–622, pl. 34, fig. 4; Bouchet and Warén 1986: fig. 1160; Weil *et al.* 1999: 46).

DISCUSSION

More than half of all known species of Nystiellidae have been reported from the Atlantic Ocean (Clench and Turner 1952, Rex and Boss 1973, Bouchet and Warén 1986, Weil *et al.* 1999, Forcelli 2000, García 2005, 2011, Rios 2009, Andrade *et al.* 2011). However, many species from the Indo-Pacific region remain undescribed (Bouchet and Warén 1986: 481, Dr. Emilio F. García, pers. comm., September 2012). The greater species richness reported in the northeastern Atlantic is the apparent result of greater collection efforts in the deep-sea in this region (Jeffreys 1877, 1883, Dautzenberg and Fischer 1896, Sykes 1925, Dautzenberg 1927, Bouchet 1977, Beu 1978, Taviani 1984, Bouchet and Warén 1986, Weil *et al.* 1999). Five genera of nystiellids are known from the Atlantic Ocean, but only *Iphitus* Jeffreys, 1883 has not yet been collected in the South Atlantic. The scarcity of records of nystiellids from the deep-sea of the South Atlantic is most probably due to the small sampling effort in the region.

A total of 14 nystiellid species have been listed for the Atlantic coast of South America (Table 1), which is an area with a great potential for the discovery of species new to science.

The genus *Eccliseogyra* is well represented in deep waters of the northeastern Atlantic, with six species (Bouchet and Warén 1986, Weil *et al.* 1999). Only *E. nitida* (Verrill and Smith, 1885) and *E. brasiliensis* Garcia, 2011 have been studied on the Atlantic coast of South America (Brazil). Herein we increase the number with the addition of *E. maracatu* n. sp. and *Eccliseogyra* sp., both inhabiting the Brazilian coast.

Andrade *et al.* (2011) recorded three species of *Opaliopsis* Thiele, 1928 from the Brazilian coast but did not locate the material for *O.* cf. *atlantis* (Clench and Turner, 1952) and *Opaliopsis* sp. identified by Miyaji (2004) in southeastern Brazil. A specimen of *Opaliopsis atlantis* was collected on the continental slope off the state of Pernambuco (690 m) during the REVIZEE/Score-Northeast Program (2000) and has been included in a recent review of the genus from Brazil (Andrade *et al.* 2011: 1562 [MNRJ 15499]).

Miyaji (2004) also identified (but did not characterize, illustrate and/or discuss) *Narrimania azelotes* (Dall, 1927) and *Narrimania* cf. *concinna* (Sykes, 1925) for southeastern Brazil based on material collected during the REVIZEE/Score-Central Program.

Only two species of *Papuliscala* have been recorded from the western Atlantic – one in the Caribbean (Watson 1886,

Weil *et al.* 1999) and one in sub-Antarctic waters (Castellanos *et al.* 1987). Although Rosenberg (2009) reports *P. annectens* (Powell 1951) from off the Falkland Islands, Bouchet and Warén (1986: 547) have reassigned this taxon to *Gregorioiscala* Cossman, 1912. The genus is recorded here for the first time in Brazil.

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Observations on the biology and sclerochronology of *Turritella leucostoma* (Valenciennes, 1832; Cerithioidea: Turritellidae) from the Gulf of California

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Abstract: Fossil and Recent shells of *Turritella leucostoma* (Valenciennes, 1832) are common in the Northern Gulf of California and, during winter months, living specimens can be found populating tidal flat environments. At San Felipe, Baja California, Mexico, *T. leucostoma* preferentially inhabit the sediment near the base of tidal channels and the low tide beach, where a total of 45 live specimens were gathered and documented. Most specimens were found in feeding position with the aperture exposed at the sediment surface and the apex pointed down at a low angle into the sediment. They act as predominantly stationary semi-infaunal active suspension feeders. Actively moving specimens were encountered during day and night low tides. The size distribution of the shells is narrow and no juveniles occur on the San Felipe tidal flat. Analysis of $\delta^{18}O$ performed on three shells shows variations of up to 3.4‰ attributable to seasonal changes in water temperature, and indicates longevity of 1.5–2 years and relatively constant growth rates. The carbon isotopic composition of the analyzed shells suggests that the individuals moved from a deeper environment to a tidal flat environment after their first year of life where they may have been subject to a change in diet and ambient dissolved inorganic carbon composition and/or began to incorporate more isotopically-light respiratory carbon into the shell.

Key words: Stable isotopes, vital effects, tidal-flat, turritellines

Turritelline gastropods (family Turritellidae, subfamily Turritellinae, mostly *sensu* Marwick 1957) are important constituents of both Recent and fossil (Cretaceous and Cenozoic) benthic assemblages. At least 1800 species names have been proposed for the group, which probably translates to around 100 living and 400 valid fossil species (Allmon 2011). Their frequently gregarious behavior and potential for mass colonization of the seafloor can have dramatic impacts on coastal ecosystems (Bax *et al.* 2003). Turritellines today occur in a range of environments (Allmon 1988, 2011). They live from intertidal to 1500 m water depth although they are most common between 10 and 100 m. They inhabit waters from the Arctic to the tropics, in water temperatures ranging from 2–30 °C, although the group as such seems to prefer moderate temperatures (< 20 °C). They are primarily animals of normal marine salinities, but several species live in salinities well below 35 psu. They apparently do not tolerate hypersaline conditions. They are found on many different types of substrates ranging from silt to gravel and even rock surfaces in a number of hydrodynamic regimes including the high-energy surf zone and reef environments. They have an epifaunal or shallow infaunal mode of life and are commonly ciliary suspension feeders. Some, however, are mucus string feeders, some may be exclusive or facultative deposit feeders, and others apparently change the dominant trophic mode according to seasonal phytoplankton availability. Reproduction is seasonal for all species for which it is known. Many turritellines remain stationary for long periods of time and they are found in a variety of life positions. They can, however, also be highly active and certain species are believed to "migrate" hundreds of meters or kilometers during days or weeks, activity apparently being greatest at night (Allmon 1988). Turritellines are preyed on by fish, stingrays, naticid and muricid gastropods, bulloid opisthobranchs, decapod crabs, starfish and octopus (Dudley and Vermeij 1978, Allmon 1988, 2011, Allmon *et al.* 1992, Bax *et al.* 2003).

Little is known about the biology of individual species. This paper is part of a series of reports (Allmon *et al.* 1992, Allmon *et al.* 1994, Waite and Allmon submitted) that provide information on habitat and biology of Recent turritelline species, which should aid researchers attempting paleoenvironmental reconstructions based on fossil turritelline occurrences. Here data on the modern turritelline species *Turritella leucostoma* (Valenciennes, 1832) in the Gulf of California is presented. Turritellines are common in the northern Gulf of California and have been reported especially from the mainland side. *Turritella anactor* (Berry, 1957) and *T. leucostoma* are reported from the western side of the Gulf (G. Dietl pers. comm., M. Tellez pers. comm.) while *T. leucostoma* and *T. gonostoma* (Valenciennes, 1832) co-occur in Pleistocene to Recent deposits on the mainland

coast of Sonora (Allmon *et al.* 1992, Tull and Boehning-Gaese 1993). While several studies have focused on *T. gonostoma*, especially from the tidal flat of Bahía la Cholla (Choya Bay) near Puerto Penasco, Sonora, Mexico (Fig. 1; Allmon *et al.* 1992, Cadee *et al.* 1997, Walker 1998) little information on the biology of *T. leucostoma* has previously been published. Here the available literature is reviewed and complemented with observations on several live individuals of *T. leucostoma* from San Felipe, Baja California, Mexico. A general characterization of the environment is also provided. The data set is completed by observations on dead specimens from Bahía la Cholla.

SYSTEMATICS AND DESCRIPTION

The following is a review of the literature of the systematics on *T. leucostoma*:

Family Turritellidae (Lovén, 1847)

Subfamily Turritellinae (Lovén, 1847)

Genus Turritella (Lamarck, 1799)

The generic-level systematics of the turritellines is, at best, inconsistent and problematic (Allmon 1996). Here we use the genus "*Turritella*" *sensu lato*.

Type species: *Turbo terebra* (Linnaeus, 1758). Recent, Indo-Pacific.

***Turritella leucostoma* (Valenciennes, 1832)**

Turritella tigrina (Kiener, 1843)

Turritella imbricata (Menke, 1847; non Lamarck)

Turritella cumingii (Reeve, 1849)

Turritella dura (Mörch, 1860)

Common names: Tiger Turret, White-mouthed Turritella

Type locality: Coast of Acapulco, Mexico.

Distribution: Northern Gulf of California to Panama (Keen 1971), possibly as far south as Colombia (Rosenberg 2012), northern Peru or even Chile (Jay 1850).

It is known to occur from the intertidal down to 40 m water depth (Keen 1971).

Shells can reach at least 120 mm in length and probably about 24 mm in basal diameter with up to 26 whorls. Each whorl is contracted just above the suture bearing a series of 4–5 riblets on each whorl (Keen 1971). On behalf of the rather small operculum, the animals can withdraw relatively far up the shell and vacate the ultimate and penultimate whorls (Fig. 2). The diameter of the operculum in an adult specimen is approximately 6 mm, which is about half the diameter of the aperture. It is flexible, shows concentric growth rings and has a jagged periphery. When withdrawn, the animal occupies about 8 whorls so only the lowest 10-12 whorls are actually inhabited while the upper whorls are vacated in adult animals and sealed off with secondary septa (Andrews 1974). The shell is cream colored with blackish brown spots

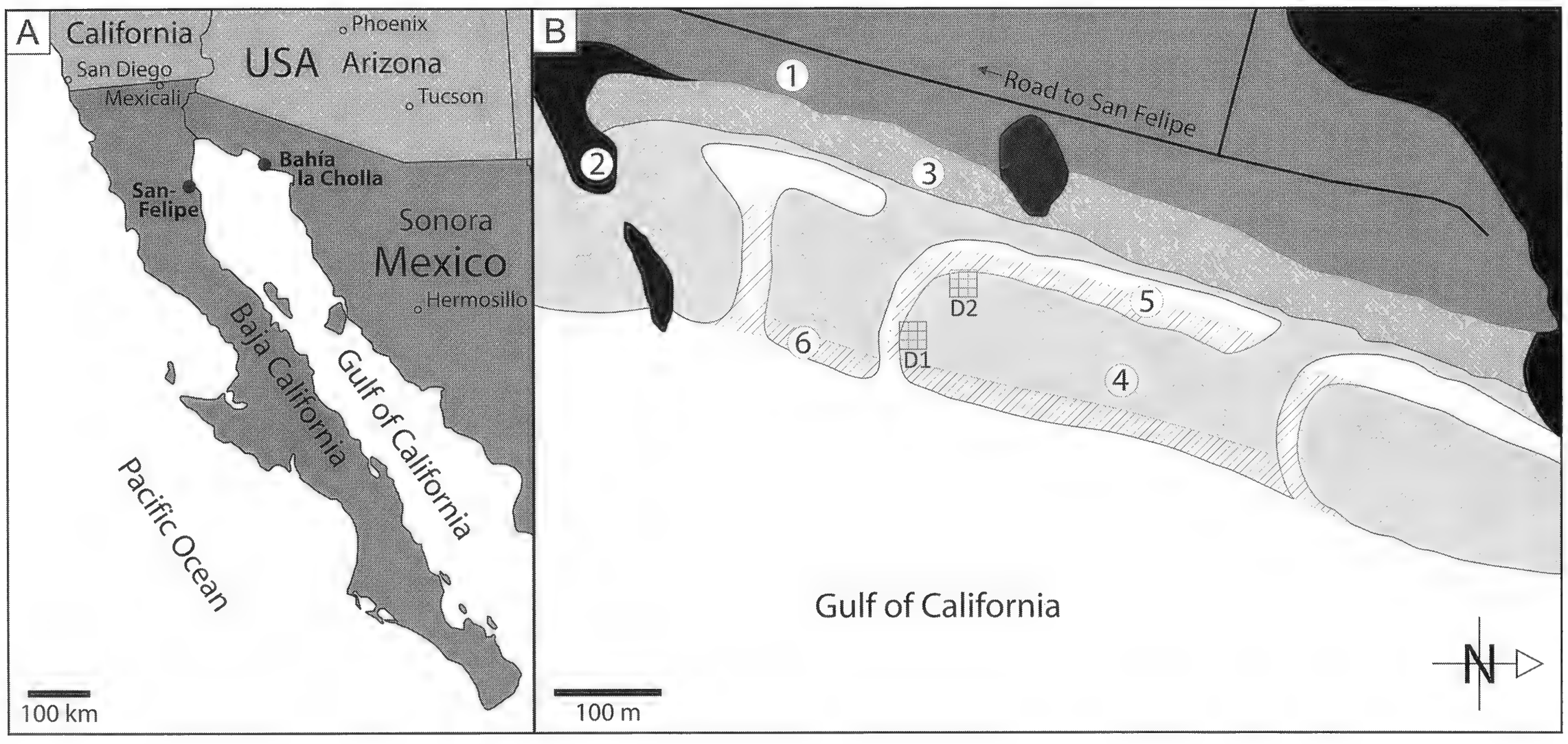

Figure 1. Map of the study area. **A,** Overview of the Baja California peninsula and the Gulf of California showing the locations of study. **B,** Detailed map of the studied tidal flat at San Felipe. 1) land, 2) rocks, 3) high-tide beach, 4) sand bank, 5) tidal channel, 6) low-tide beach. D1 and D2 are the excavation sites. The hatched areas delimit the habitats where *Turritella leucostoma* was found.

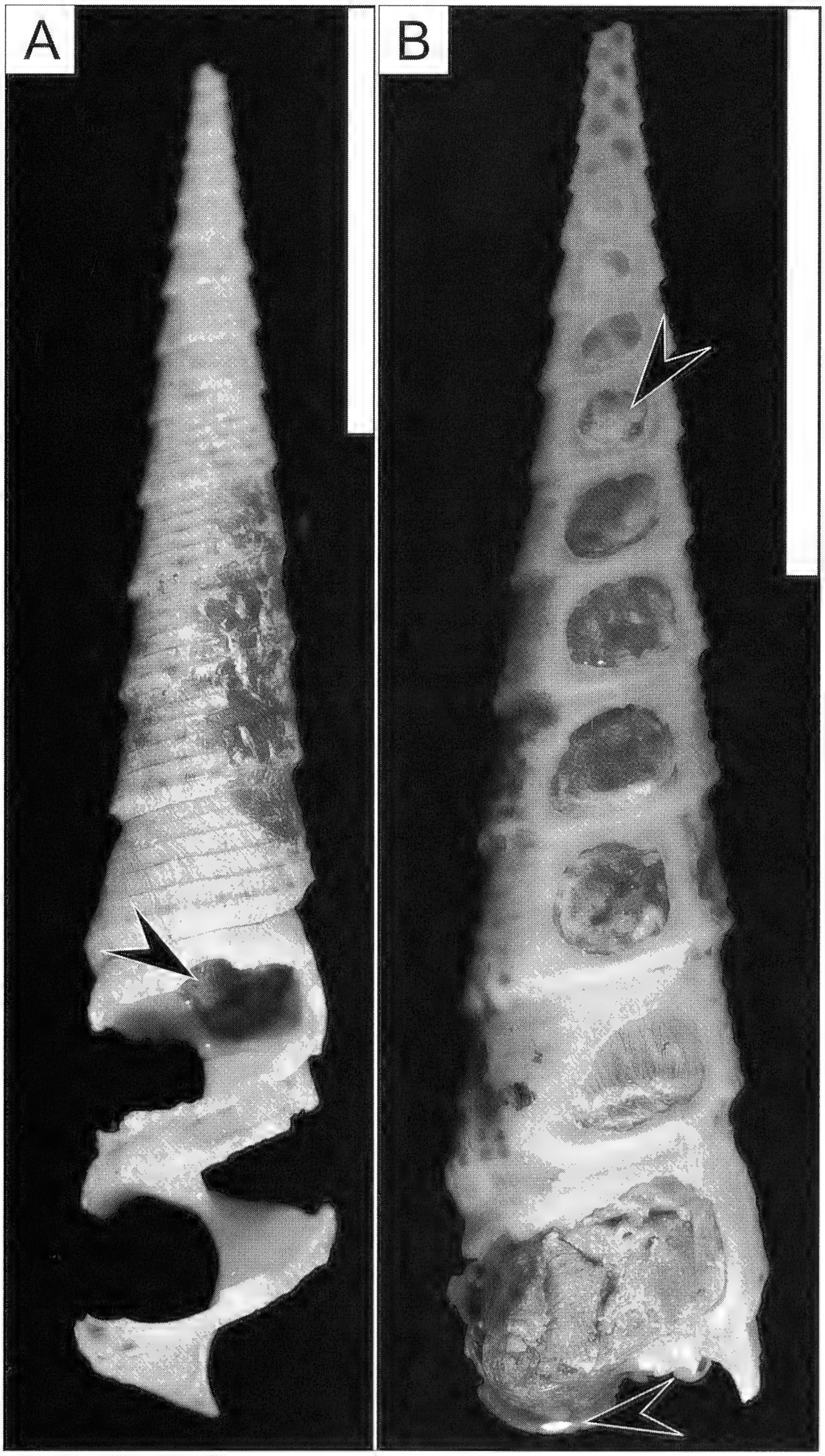

Figure 2. Photograph of a preserved specimen of *Turritella leucostoma* from San Felipe. **A,** Lowest two whorls of the shell broken open to expose the operculum (arrow) of the animal when completely retracted into the shell. **B,** Same specimen as in A, with the upper whorls cut open, exposing the extent of the spire, which is occupied by the animal (arrows). Scale bars 2 cm.

and longitudinal flames. The aperture has been remarked upon for both its whitish color and its round cross section (Valenciennes 1832, Kiener and Fischer 1873).

GEOLOGICAL AND GEOGRAPHICAL SETTING

The town of San Felipe is situated on the east coast of the Baja California peninsula 200 km south of the international border between the U.S.A. and Mexico at 31°1′37″N, 114°49′49″W (Fig. 1). The climate is very dry with annual precipitation around 125–224 mm. Rainfall is biseasonal with maxima occurring in summer and winter. Winds are predominantly from the northwest between November and May and southwest during the remainder of the year (Douglas *et al.* 1993, Ortega-Guerrero *et al.* 1999). Winds during the surveying period were strong and mainly from the northeast. Mean annual temperatures are around 18–20 °C with maxima and minima reaching 46 °C and 1 °C, respectively. Roden and Groves (1959) reported average sea surface temperatures (SST) at Puerto Penasco of 14.9 °C in January and 31.2 °C in August. Robinson (1973) recorded mean monthly surface temperatures between 15 °C in February and 29.5 °C in August for the waters around San Felipe. Net evaporation in the northern Gulf equates to approximately 0.95 m y^{-1} with the most pronounced values occurring in late summer (Bray *et al.* 1988). Evaporation exceeds precipitation and run-off throughout the year in the northern basin, along the coast of Baja California. In the Gulf, salinities range from 35.58–34.93 psu with salinities exceeding 36 psu only found on the shallow shelves of the Colorado River delta. Substantial tidal mixing of northern Gulf waters is thought to be responsible for the normal salinity despite the high evaporation. (Robinson 1973, Bray *et al.* 1988). Tides are semidiurnal and tidal range varies strongly. During the study month, tides varied from about 7.6 m at spring tide to about 40 cm at neap tide.

MATERIALS AND METHODS

Field observations

A tidal flat and beach at San Felipe was documented and analyzed for coast morphology and faunal abundance and diversity with a special focus on the population of turritelline gastropods. The particular stretch of beach was initially chosen because it proved to be the only stretch of beach in the vicinity of north San Felipe where dead shells were observed on the beach (Fig. 1). A total of 45 live and 23 well-preserved dead specimens were collected from the beach and tidal flat

during a 4-day spring tide period between 17–20 February 2011. Studies were performed by day and night during the low tide periods by documenting transects parallel and perpendicular to the beach line. Transects were conducted by walking from the beach to the low-tide water line, or from tidal channel to tidal channel, respectively. Documented elements included topography; sediment composition, bottom stability (overgrowing organisms) and water content; as well as benthic faunal elements. Specimens of *Turritella leucostoma* were measured in length and basal width and documented and catalogued with regard to their location and position on the flat or beach, their state of preservation and any unusual or interesting features. After examination, most live specimens were returned to the field while seven specimens were stored in alcohol and brought back to the lab for later examination. Additionally two excavations over an area of 9 m^2 to a depth of 7 cm were conducted to estimate diversity and frequency of turritellines and accompanying shallow infauna. Temperature measurements were made with a dive computer.

Isotope sampling

In the absence of direct observations, analysis of annual variations in oxygen stable isotopic composition of shell carbonate can be applied for the assessment of age and growth rates of gastropods (Allmon *et al.* 1992, 1994, Jones and Allmon 1995). Carbon isotopic data can provide valuable information on salinity and food composition (Gentry *et al.* 2008). These techniques have been applied to three shells of *Turritella leucostoma* in the present study.

Two of the analyzed specimens were collected alive from San Felipe, Baja California, Mexico on the 19th of February 2011, and the third was collected dead from Bahía la Cholla, near Puerto Peñasco, Sonora, Mexico on the 20th of March 2011 as a comparison. The first two specimens were approximately the same size, being (specimen *Turritella leucostoma* 1 (*Tl1*): 16 whorls, complete aperture, 2 uppermost whorls of teleoconch missing) 95.0 mm and (specimen *Turritella leucostoma* 2 (*Tl2*): 21 whorls, complete aperture, protoconch missing) 87.7 mm, respectively, in length. The specimen from Sonora was slightly larger with a total length of (specimen *Turritella leucostoma* 3 (*Tl3*): 24 whorls 2 uppermost whorls missing, last half whorl of aperture missing) 117.3 mm. The sampling yielded 56 separate drilled powder samples (*Tl1*: 17, *Tl2*: 22, *Tl3*: 17). In *Tl1* and *Tl2* the uppermost 5 whorls were combined into two samples and from the lowermost 4 whorls two samples were extracted per whorl. One sample was extracted from each of the remaining whorls. In *Tl3* the uppermost 6 whorls were not sampled at all and one sample per whorl was extracted from all other whorls with one additional sample being taken at the repair mark after a scar, 5.5 cm from the aperture.

For the preparation of aragonite powder samples for isotope analysis, shell surfaces were cleaned with hydrochloric acid and rinsed with distilled water and alcohol. Isotope samples were extracted by grinding shallow grooves into the outer shell layer of each whorl manually with a dental drill. Outer shell layers were targeted to avoid secondarily thickened shell layers, which are internally accreted later in life.

Isotope analysis

About 400 µg of powdered sample were flushed with Helium (120 ml/min for 5 minutes) and then reacted with 100% phosphoric acid offline for 24 hours at 50 °C before analysis, based on the method of McCrea (1950). All samples were analyzed for their carbon- and oxygen-isotopic composition at the Cornell Isotope Laboratory (COIL). The C- and O-isotope composition was measured using a Thermo Scientific Delta V isotope ratio mass spectrometer (IRMS) interfaced to a Thermo Finnigan Gas Bench II, in continuous flow mode using He carrier gas. The method used four measuring peaks for each sample. Average values of the samples measured were normalized using two international standards and checked with an internal standard. Results are expressed relative to the Vienna Peedee belemnite (VPDB) carbonate standard by calibration to the NBS-18 ($\delta^{13}C = -5.01‰$ and $\delta^{18}O = -23.20‰$) and NBS-19 ($\delta^{13}C = +1.95‰$ and $\delta^{18}O = -2.20‰$) international reference standards. Values were obtained with precisions of: NBS-18 (0.07‰ for $\delta^{13}C$ and 0.1‰ for $\delta^{18}O$) and NBS-19 (0.06‰ for $\delta^{13}C$ and 0.09‰ for $\delta^{18}O$).

RESULTS

Description of the environment

The studied tidal flat is located to the north of the town of San Felipe and is bounded to the south by the lighthouse and to the north by a mountain that reaches out on to the tidal flat and marks the beginning of the rocky shoreline (Fig. 1). This stretch is about one kilometer in length and at low tide approximately 200 m in width (Fig. 3). The beach and flat are predominantly composed of sand although pebbles and coarse gravel intermixed with shell debris occur locally in a 4 m wide strip at the foot of the high tide beach as well as around the rocks at the north and south ends of the flat. At low tide the flat is drained by three major tidal channels. The largest of these is the central channel, which drains a major portion (~80,000 m^2) of the flat and is about 3 m wide and 20 cm deep at low tide. The tidal channels run parallel along the beach and then swing round and flow out more or less perpendicular to the waterline (Fig. 3). The north channel is comparable but has slightly less capacity draining approximately 60,000 m^2 of tidal flat and the south channel only drains a relatively small area (~45,000 m^2). The tidal channels

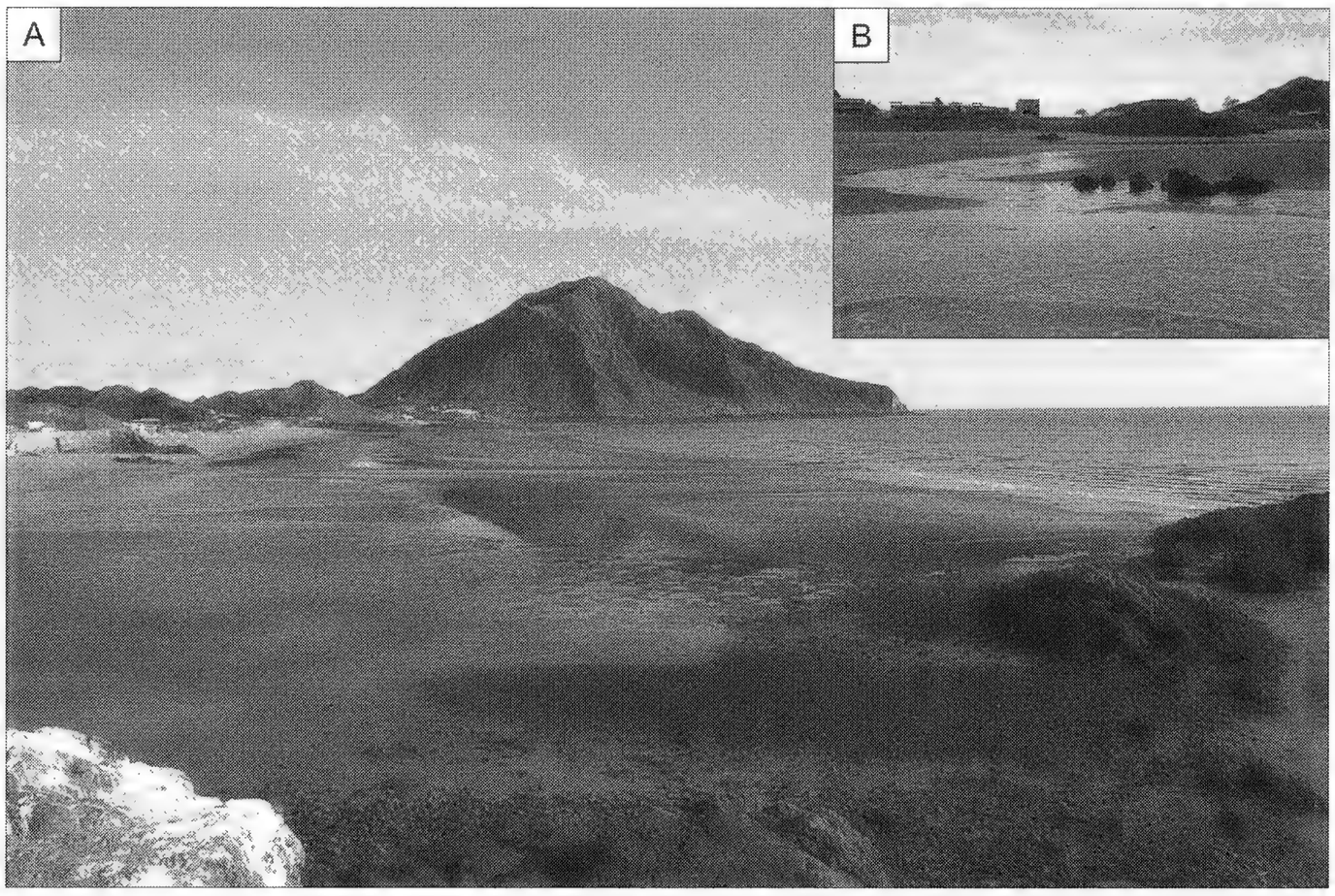

Figure 3. Photograph of the studied tidal flat. **A,** Overview of the tidal flat at low tide. Taken looking to the north. Southern rocks in the foreground compare to Fig. 1. **B,** Detailed view of the lower part of the central tidal channel where it runs perpendicular to the beach.

delimit the major sand banks between them. A schematic transect across the flat is shown in Figure 4. At low tide the beach is relatively steeply inclined and dips down about five meters to the level of the upper tidal flat. For the most part, one of the major tidal channels runs along the foot of the beach, separating the beach from the sandbank—that rises about a meter above the tidal channel—and draining both beach and sandbank through numerous smaller tributaries.

The sandbank is inhabited by a number of bivalves (Donacidae (Fleming, 1828), Tellinidae (Blainville, 1814), Veneridae (Rafinesque, 1815), Mactridae (Lamarck, 1809), Glycymerididae (Newton, 1922), Cardiidae (Lamarck, 1809)) gastropods (Naticidae (Auilding, 1834), Muricidae (Rafinesque, 1815), Turridae (H. Adams and A. Adams, 1853), Trochidae (Rafinesque, 1815), Terebridae (Mörch, 1852), Olividae (Latreille, 1825)), box crabs (*Hepatus*), irregular echinoids (*Mellita*) and annelid worms. Naticid and olivid gastropods were frequently found with anemones attached to their shells. The northern rocks are made up of large blocks in the dm^3 range, which are relatively barren with only a few oysters and barnacles encrusting them and hermit crabs are infrequent.

The southern rocks are outcrops of heavily weathered metamorphic rocks about 4 meters high. At the base of these rocks hot springs indicated by the constant rise of bubbles flow out of the rock and at least during low tide appreciably warm the water. Between the southern rocks and the southern tidal channel the sediment is more fine-grained and has a higher proportion of mud compared to the rest of the flat. The mud and water seeps together make this part of the tidal flat thixotropic. The southern rocks are covered by a rich encrusting fauna consisting of chitons, vermetid gastropods, coralline algae, serpulids worms, barnacles (*Balanus*), oysters (*Crassostrea* Sacco, 1897), sponges (*Pseudosubterites*), tunicates (*Botrylloides*) and small corals (*Pocillopora*). On and between the rocks large numbers of hermit crabs occur and hundreds to thousands of colonized turritellid shells in various states of encrustation and decay were observed.

During the study 45 live specimens of *Turritella leucostoma* were recorded on the tidal flat. Additionally 23 well-preserved dead specimens were collected from the flat and the beach. One shell of *Turritella anactor* was found dead on the beach.

Turritella leucostoma was found preferentially inhabiting the lowest areas of the sandbank on the distal side of the tidal channels and the low tide beach where it is only exposed to sub areal conditions at spring tide. *Turritella leucostoma* was found on the sediment surface only in rare cases and usually it was shallowly buried with only a small part of the spire exposed at the surface (Fig. 5). When under water, it remains stationary, right below the sediment surface and forms two small holes over the otherwise covered aperture.

The sediment on the tidal flat is characterized by an oxidized superficial layer 2–3 cm

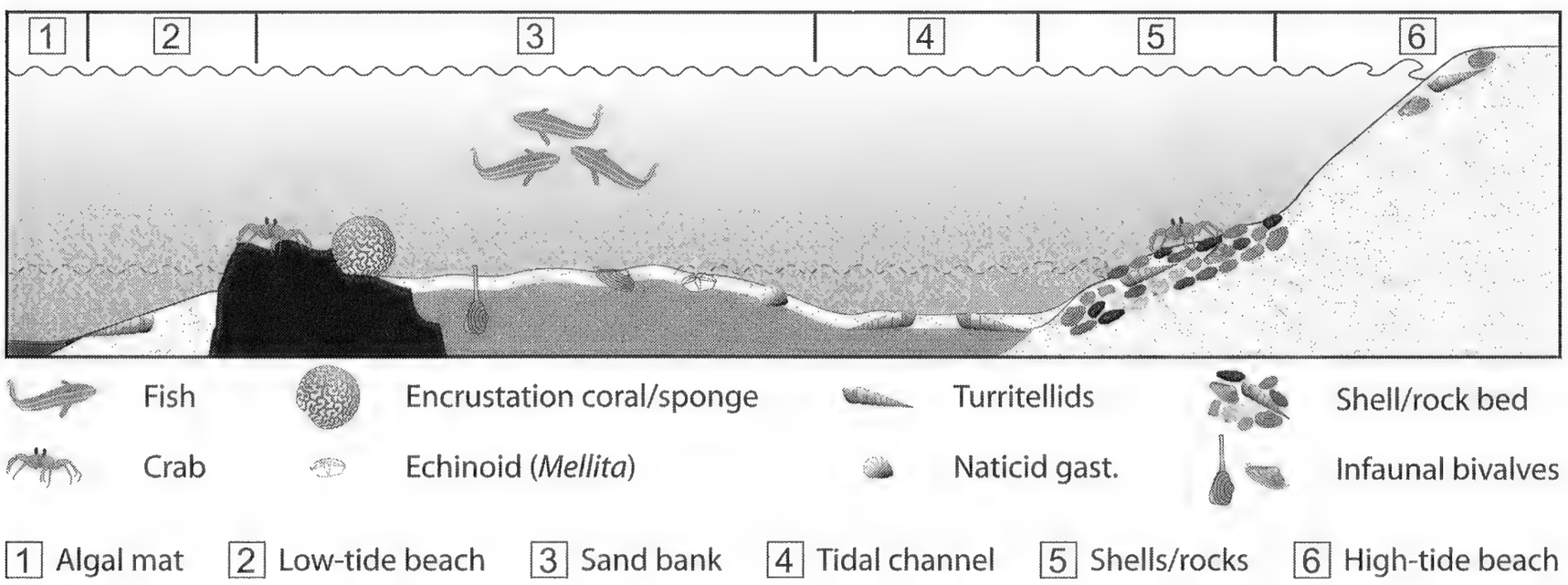

Figure 4. Schematic generalized transect across the tidal flat depicting the major distinguishable zones and common faunal elements. Dashed line corresponds to mean low tide line.

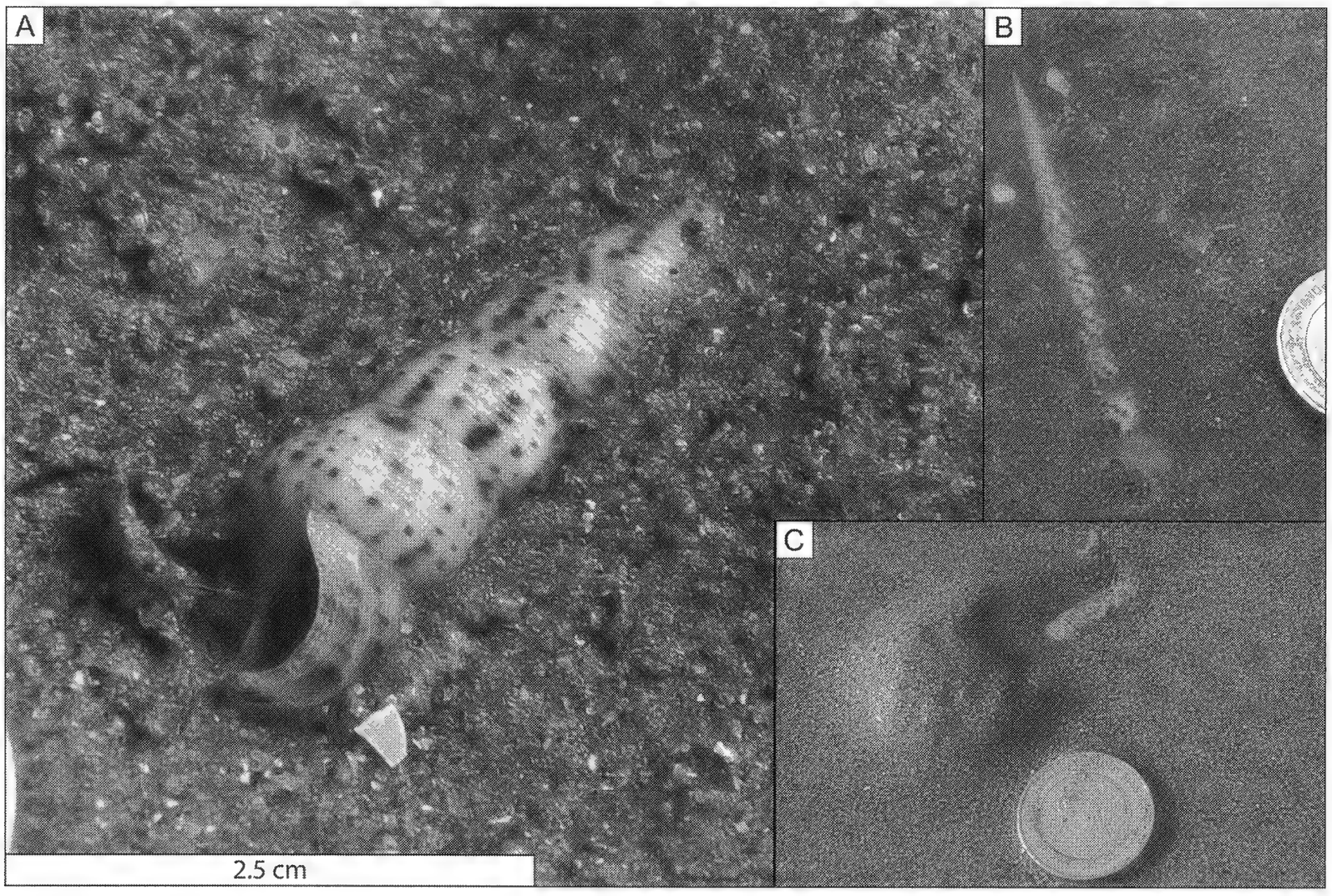

Figure 5. Photographs of positions of *Turritella leucostoma* on the tidal flat at low tide. **A,** Feeding position with aperture and some of the lower spire exposed and aperture oriented oblique to the surface. **B,** Feeding position with part of spire buried. **C,** Moving/burrowing position with most of the spire buried. Coin for scale is 2.7 cm in diameter.

thick of grey sand overlying a darker, organic-rich, almost black layer below. Beyond the low tide beach, where the sea floor is permanently covered by water, the sediment is stabilized by an algal mat, which produces a firm ground and no turritellines were observed there (Fig. 6). Turritellines were only found living and feeding in the tidal channels and on the low tide beach in water of 16 °C. Most of the live specimens (n = 32) were found semi-embedded in the sediment in the main channel. A few were found on the low tide beach of the main sand bank (n = 7), along the north channel (n = 3) and in the south channel (n = 1). One was found washed up on the beach, and one was found lying on the surface of the southern sand bank. The size distribution of the dead and live specimens is presented in Fig. 7. No shells smaller than 46 mm were encountered dead or alive on the beach or tidal flat. Of the 23 dead specimens, 13 had been successfully drilled by predatory gastropods and 2 had been unsuccessfully drilled.

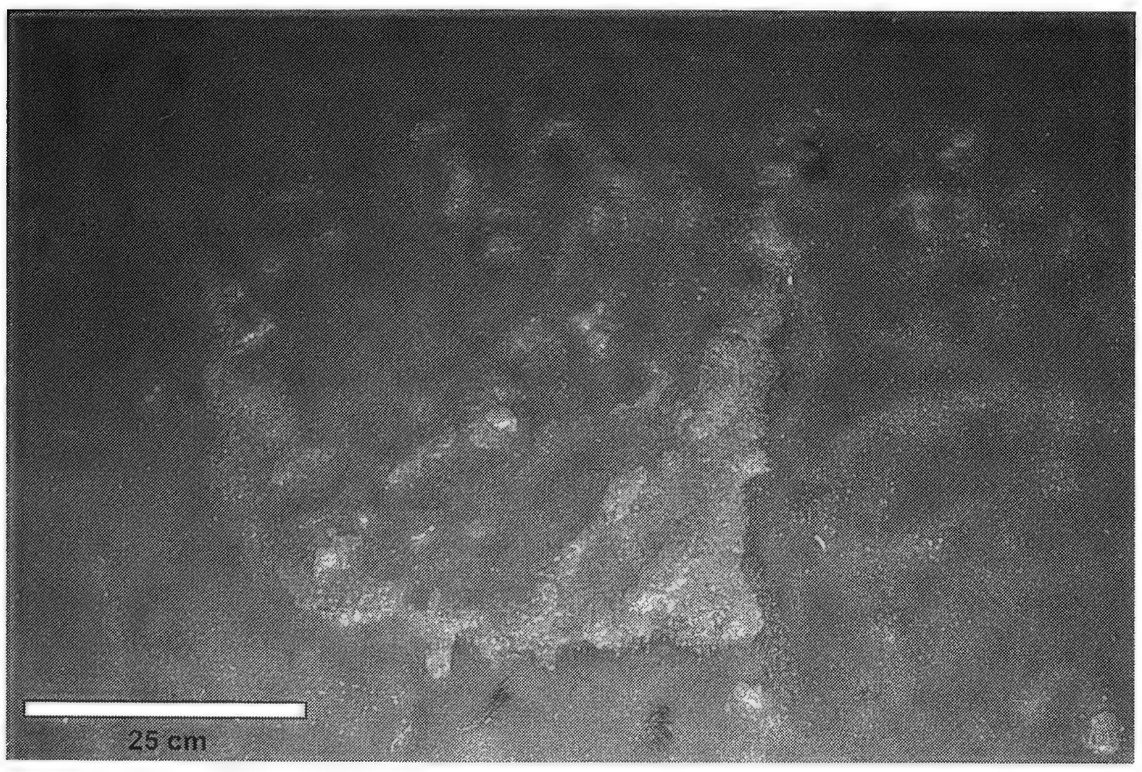

Figure 6. Photograph of the algal mat covering the sea floor surface beyond the low tide beach and presumably preventing turritellines from burrowing. The mat stabilizing the sediment and produces a firm ground, which is being eroded due to wave action at extreme low tide.

In this study, repair scars were observed in both live (n = 5) and dead specimens (n = 9). Damage at the present aperture was observed in one live specimen and one dead specimen. Excavation D1 exposed: 2 small fish, 2 olive snails, 3 sand dollars, 1 naticid snail, 2 worms and 4 clams. Excavation D2 exposed: 3 worms one muricid snail and 6 clams. Both excavations were conducted on the low distal flank of the central tidal channel next to where a specimen of *T. leucostoma* had been found.

Oxygen and carbon stable isotopes

The stable oxygen and carbon isotope profiles for each specimen are plotted in Figure 8 in standard fashion, with lighter (depleted) values toward the top. $\delta^{18}O$ minimum values reach around -1.45‰ and maximum values extend to around +1.95‰, in the San Felipe specimens (*Tl1* and *Tl2*) while the specimen from Sonora (*Tl3*) records slightly more depleted $\delta^{18}O$ values between -1.68‰ and +1.14‰. The $\delta^{13}C$ values show similar ranges and less variability than the oxygen values (Table 1).

DISCUSSION

Environment and size distribution

Turritella leucostoma is the only species of turritelline gastropod that currently inhabits the studied tidal flat. Although one shell of *Turritella anactor* was found, the shell was not fresh and no further evidence of the presence of *T. anactor* on the tidal flat was encountered. *Turritella anactor* is, however, described in the literature from San Felipe (Keen 1971) and is known on the west coast of the Gulf from tidal flats

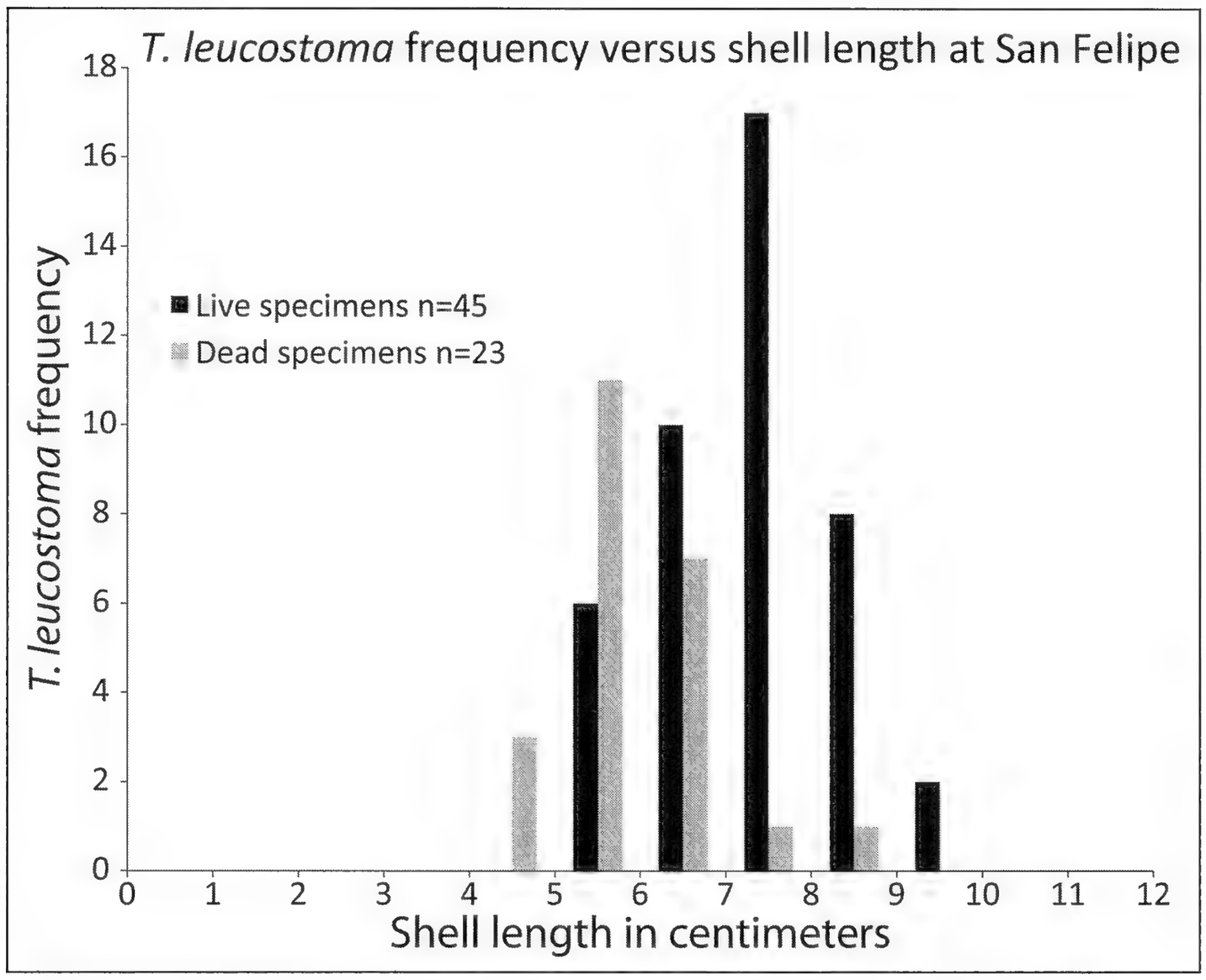

Figure 7. Graph of the measured population of well-preserved dead and live *Turritella leucostoma* shells documented on the studied tidal flat.

further north (G. Dietl pers. comm. 2012) and shell middens (M. Tellez pers. comm. 2011). In comparison to other turritelline populations in subtropical tidal flat environments described in the literature (Allmon *et al.* 1992, Waite and Strasser 2011), *T. leucostoma* is infrequent at San Felipe in February.

From the distribution of the specimens on the flat it is concluded that *Turritella leucostoma* is selectively colonizing habitats on the tidal flat, and that the base of the tidal channels is their preferred microenvironment. They share this habitat with several other benthic macrofauna including annelid worms, naticid, muricid and olivid gastropods and glycymeridid, venerid, mactrid and cardiid bivalves, as well as common sand dollars. The common occurrence of *T. leucostoma* at the base of the tidal channels, where they are regularly subjected to moderate to strong currents, may be due more to the higher suspended food supplies provided by such currents than to any substrate preference (c.f. Allmon *et al.* 1994).

The stabilizing algal mat beyond the low tide beach presumably prevents turritellines from burrowing. The sand bank itself may be too high above the low tide waterline and be exposed to subaerial conditions for too long to be suitable for turritellines.

The specimens on the flat were usually buried in the sediment with the aperture positioned immediately under the surface similar to *Turritella gonostoma* (Allmon *et al.* 1992; Fig. 5). In *T. gonostoma* and *T. duplicata* (Linnaeus, 1758) this behavior has been shown to be associated with a method of active suspension feeding with the two holes forming in the sediment cover above the aperture as openings for the in- and excurrent of the filtering process (Waite and Strasser 2011).

The feeding holes although encountered during this study, were unfortunately not captured on film satisfactorily. Other positions included lying completely exposed on the surface with the operculum loosely closing the aperture, apex sticking down in the sediment, or moving across and through the sediment (Fig. 5).

The size distribution of the dead and live specimens of the measured population suggests that only adults are present on the tidal flat (Fig. 7). No shells smaller than 46 mm were encountered dead or alive on the beach or tidal flat. No living shells smaller than ~55 mm were observed, and the smaller shells in the dead assemblage are ones that show minor damage to apex and aperture accounting for some loss in shell length. Similar observations on the size distribution of shallow water populations of different species of turritellines have been made by other authors (Allmon 1988, Walker 1998, Waite and Strasser 2011). A study on gonad development on the turritelline species *Maoricolpus roseus* (Quoy and Gaimard, 1834) in Australia revealed that maturity for both males and females is reached at sizes between 3 and 4 cm (Bax *et al.* 2003). Previous authors (*e.g.*, Allmon 2011) have suggested that the narrow size-frequency distribution in almost all turritelline assemblages indicates rapid growth of discrete reproductive cohorts. Therefore, the population of *Turritella leucostoma* studied here may represent a community of mature adults (Fig. 7). It is possible that turritellines congregate in shallow water for reproduction (Allmon 2011). *Turritella gonostoma* is known to reproduce during winter in shallow surface waters on tidal flats at Bahía la Cholla on the opposite side of the Gulf (Allmon *et al.* 1992, Cadée *et al.* 1997). Unfortunately our observations represent only a snapshot in time and the abundance and distribution of the turritellines may change with the season. Discussion with local fishermen suggests that turritellines can be found in shallow waters all year round. Since no juvenile individuals were encountered dead or alive on the flat, however, it must be concluded that younger individuals do not live in shallow waters and must spend their early ontogenetic stages in deeper water off shore, as suggested by Allmon (1988).

Oxygen isotopes and age

Estimates of life spans for different species of turritellines range between 2–3 and 10–15 years (Allmon 1988). In the

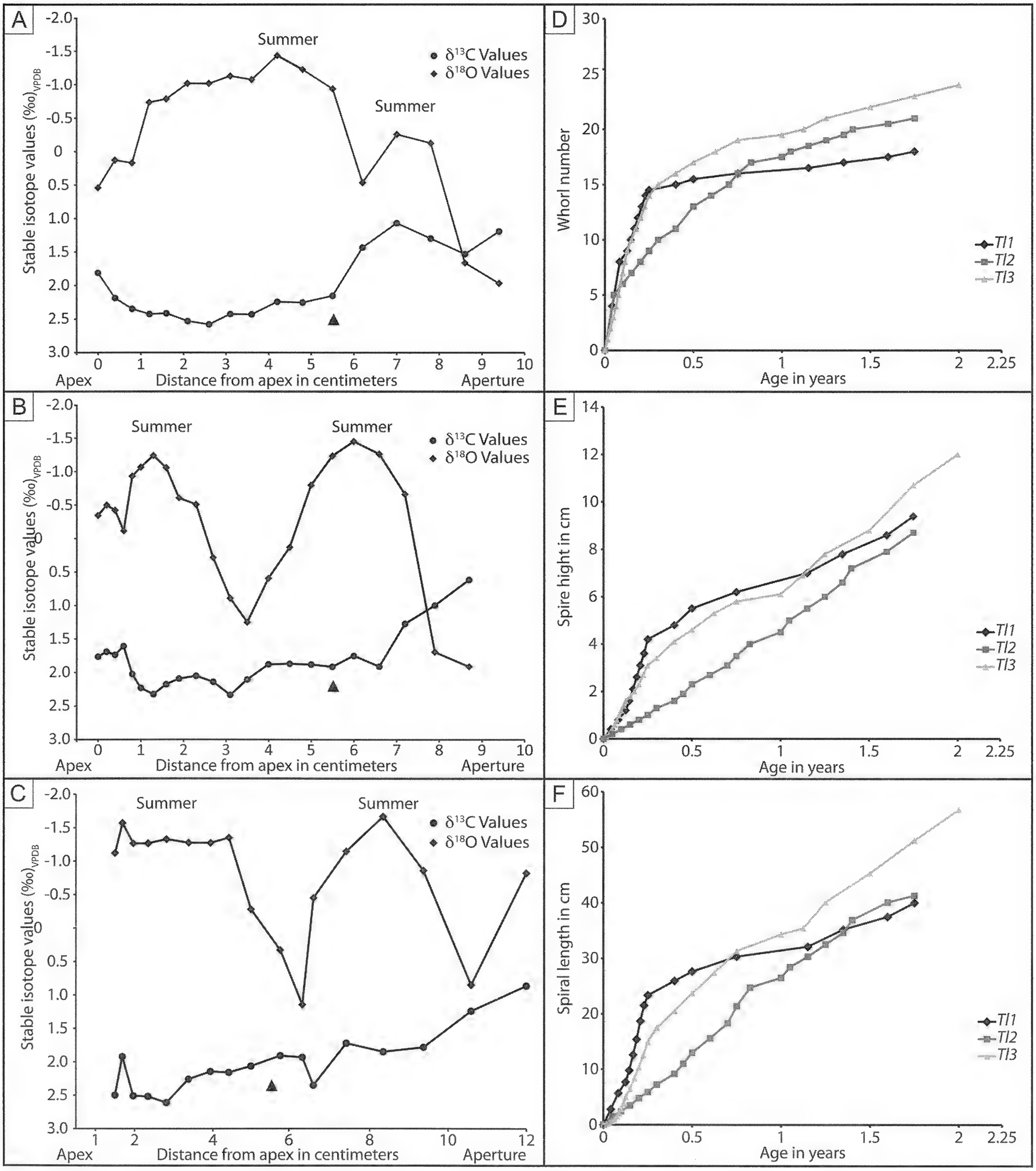

Figure 8. Results from the isotope analysis. **A,** $\delta^{13}C$ and $\delta^{18}O$ stable isotope profiles through specimen *Tl1*. **B,** $\delta^{13}C$ and $\delta^{18}O$ stable isotope profiles through specimen *Tl2*. **C,** $\delta^{13}C$ and $\delta^{18}O$ stable isotope profiles through specimen *Tl3*. Black arrows mark the 55mm growth stage. **D,** Growth rate expressed as whorls per year. **E,** Growth rate expressed as shell length per year. **F,** Growth rate expressed as spiral length per year.

Table 1. Stable isotope values for the three measured specimens.

Isotope	Sample	minimum	maximum	range	mean
	Tl1	-1.44‰	1.97‰	3.40‰	-0.39‰
δ18O	Tl2	-1.45‰	1.91‰	3.36‰	-0.24‰
	Tl3	-1.68‰	1.14‰	2.82‰	-0.79‰
	Tl1	1.07‰	2.58‰	1.51‰	2.01‰
δ13C	Tl2	0.61‰	2.34 ‰	1.73‰	1.83‰
	Tl3	0.86‰	2.61 ‰	1.75‰	2.01‰

absence of direct observations, oxygen isotope sclerochronology has proven successful in estimating age and growth rates for different fossil and living turritelline species. Allmon *et al.* (1992, 1994) found that individuals were between 1.5–3.0 years old at the time of collection. More recent results suggest that most species live to be no more than 3–5 years old and that growth is more rapid in juvenile stages (Allmon 2011).

The $\delta^{18}O$ value of mollusc shell carbonate is a function of the oxygen isotopic composition of the ambient seawater and the temperature, at which precipitation takes place. On account of the relatively minor salinity variations (and hence water composition) in the northern Gulf, seasonal variations in water temperature will be reflected in the composition of shell carbonate (Fig. 8A–C). The significant changes in SST over the year in the northern Gulf (Fig. 9) are thought to be the driving factor for the cyclical variation recorded in ontogenetic $\delta^{18}O$ profiles in molluscs (*e.g.*, Lécuyer *et al.* 2004), hence it is possible to estimate the number of seasons a specimen has lived based on the number of annual cycles recorded in its shell.

Assuming equilibrium precipitation (Epstein *et al.* 1953, Mook and Vogel 1968) and a water composition of 0.3 per mil for the northern Gulf seawater (Dettman *et al.* 2004 and interpolated from Schmidt 1999, Rohling 2007), the aragonite paleotemperature equation of Grossman and Ku (1986), yields seasonal extremes of 14 °C and 31 °C, respectively, and a mean temperature for the growth season of 24–27 °C. This corresponds well to the records of SST in the northern Gulf (*e.g.*, Robinson 1973). Although the calculated temperature estimates from the shells match the actual measurements of the SST, it should be noted that temperature dependence on depth is not significant in the upper 60 m of the northern Gulf (Robinson 1973). On account of this, young individuals living in offshore marine habitats would still be subject to variations in temperature comparable to those at the surface, and, therefore, exhibit strong seasonal $\delta^{18}O$ variations.

Based on the interpretation that the cycles in the $\delta^{18}O$ isotopic compositions represent seasonal variations in temperature of the ambient seawater, the two specimens from San Felipe (*Tl1*, *Tl2*) were probably slightly less than two years old, having hatched and died during the cool season and having witnessed two summers. The larger specimen from Sonora was slightly older than the others and had probably completed its second year when it died.

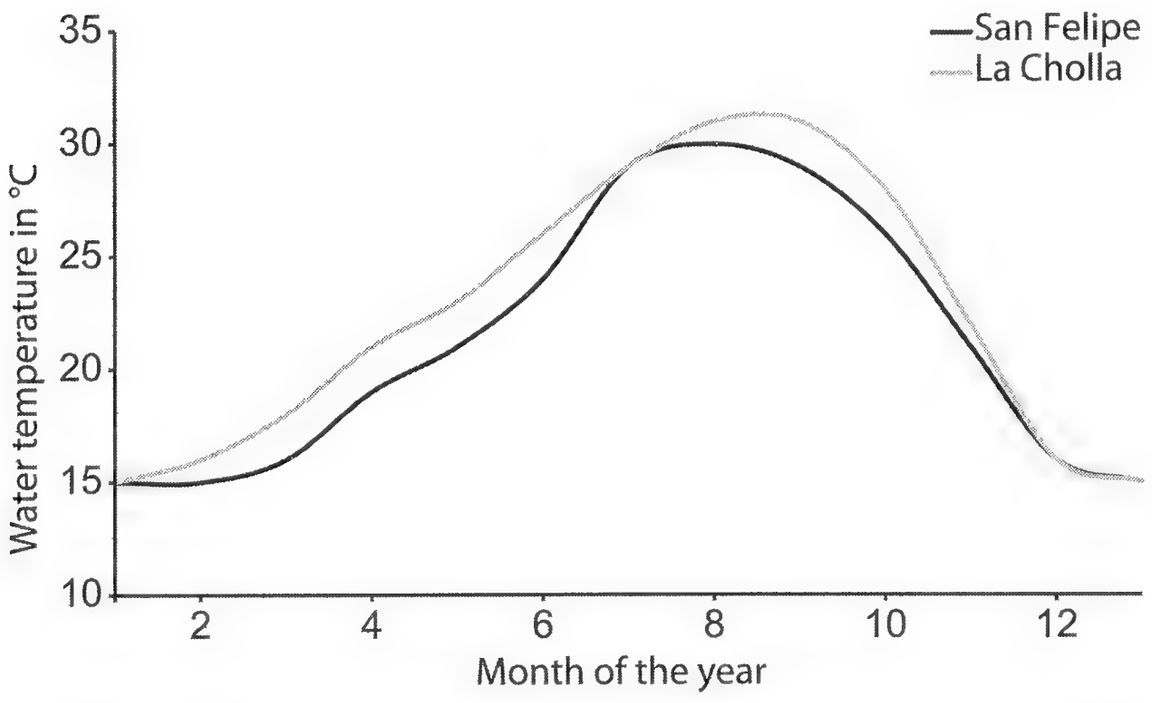

Figure 9. Mean monthly SST values for the two localities San Felipe and La Cholla (Robinson 1973).

Growth rate

For several turritelline species reported in the literature, growth is seasonal, with the most rapid growth occurring during the warmest months, particularly in the first year of life (Bax *et al.* 2003). The reduction in shell growth rate may reflect an ontogenetic decline as more investment is put into reproduction (Allmon *et al.* 1992). Other authors suggest that a relatively high growth rate in young individuals is advantageous to minimize lifetime spent in the size class most vulnerable to predation (Tull and Boehning-Gaese 1993). The limited data available for growth rate and size-maturity relations suggest extreme caution is necessary when extrapolating from one species to another.

Growth rate models were constructed by fixing summer and winter minima and maxima—and autumn and spring averages—as anchor points for age and interpolating between them. If veligers hatched in early spring then summer minima roughly corresponds to 0.25 years and 1.25 years and winter maxima to 0.75 years and 1.75 years, respectively. The results from this study do not unequivocally confirm the observation that growth is more rapid early in ontogeny. Specimen

Tl1 appears to have precipitated the initial 6.5 cm of total spire length (approximately 16 out of 18 whorls) in the first year of life. This leaves only approximately 3 cm (2 whorls) for the second year (Fig. 8D, E). Although the other two specimens show a similar growth pattern when whorl number is plotted against age (Fig. 8D), the growth rate appears more linear when spire height is plotted against age (Fig. 8E). When the spiral length is plotted against age (Fig. 8F), all the growth rate curves seem to show a reduction in slope inclination in the later ontogenetic stages although especially in specimen *Tl2* it is very slight. Finally, the mass of calcium carbonate precipitated in each year of life was estimated by cutting a shell of comparable size to *Tl1* in two, separating the lowest two whorls from the rest of the spire. The two halves—which roughly correspond to the first and second years of growth—both weighed almost exactly 4 grams, indicating that there is no change in the rate of actual shell carbonate precipitation.

Carbon isotopes and habitat

Carbon isotope data in Recent molluscs are more complex and more difficult to interpret than oxygen data because they are not only dependent on physical parameters. Aquatic molluscs incorporate carbon from different sources into the shell and so-called 'vital effects', related to changes in the biology of the animals, potentially play a significant role in the composition of the final precipitate (Grossman and Ku 1986, Lorrain *et al.* 2004, Gillkin *et al.* 2007). Isotopic changes through ontogeny, including trends to more negative values in shell $\delta^{13}C$ with age, are common in both marine and freshwater molluscs (Lorrain *et al.* 2004, Gentry *et al.* 2008, McConnaughey and Gillikin 2008). Many examples of ^{13}C-depleted isotopic compositions have been attributed to vital effects (Grossman and Ku 1986, Klein *et al.* 1996).

Kinetic vital effects in aquatic mollusc shells are favored by rapid skeletogenesis (McConnaughey 1989) and are due to slower hydration and hydroxylation of CO_2 molecules with heavy isotopes (McConnaughey *et al.* 1997). Studies of kinetic effects on aquatic mollusc shells have repeatedly been demonstrated to show no significant effect on composition (Klein *et al.* 1996, Lorrain *et al.* 2004). However, more recently Butler *et al.* (2011) have argued that kinetic effects may be responsible for observed trends to more ^{13}C-depleted values in the portions of juvenile *Arctica islandica* (Linnaeus, 1767) bivalve shells, which show the highest growth rates. Metabolic vital effects are related to the activity of metabolism and high rates of mantle metabolic pumping of respiratory CO_2 to calcification sites (Klein *et al.* 1996). Metabolic effects have been suggested to be responsible for disequilibrium shell compositions, and especially depleted $\delta^{13}C$ values in later ontogenetic stages, in different bivalves (Klein *et al.* 1996, Lorrain *et al.* 2004, Gillikin *et al.* 2007). This suggests that low metabolic activity forces incorporation of carbon from seawater with an ambient DIC signal whereas when metabolic activity is high, more respiratory (^{13}C-depleted) carbon is supplied to the calcification sites and preferentially incorporated into the shell (Chauvaud *et al.* 2011). However, it should be noted that for the well-studied, long-lived species *Arctica islandica* the metabolic contribution to the shell—at least for adult specimens—has been demonstrated to not vary significantly (Butler *et al.* 2011, Schöne *et al.* 2011, Beirne *et al.* 2012), Similarly, Keller *et al.* (2002) found no distinguishable trend to ^{13}C-depleted values in the ontogenetic data series of the bivalve species *Callista chione* (Linnaeus, 1758).

Aquatic molluscs incorporate carbon from both respired CO_2 and ambient dissolved inorganic carbon (DIC) into their shells; the isotopic composition of the shells will depend on the composition—and the relative contribution—of the individual sources (Klein *et al.* 1996, Lécuyer *et al.* 2004, Lorrain *et al.* 2004, McConnaughey and Gillikin 2008, Chauvaud *et al.* 2011). Isotopically-depleted carbon from respired CO_2 is thought to contribute only about 10% to the shells of most aquatic molluscs (McConnaughey *et al.* 1997) and their isotopic composition mainly reflects ambient DIC (Gentry *et al.* 2008, McConnaughey and Gillikin 2008, Beirne *et al.* 2012). Negative carbon isotope values in DIC should result from the oxidation of organic matter into bicarbonate ions in the ambient seawater (Lécuyer *et al.* 2004). Pore waters, in particular, are depleted in ^{13}C due to the oxidation of organic matter and this may influence the skeletal composition of infaunal molluscs (*e.g.*, Keller *et al.* 2002, Lorrain *et al.* 2004, Walter *et al.* 2007, McConnaughey and Gillikin 2008). Depleted $\delta^{13}C$ values due to runoff of fresh water with isotopically-negative DIC, associated with the decomposition of terrestrial organic matter have been found to be of secondary importance by some authors (Klein *et al.* 1996, Lécuyer *et al.* 2004). However, Gentry *et al.* (2008) demonstrated a significant covariance of $\delta^{13}C$ values and seasonal salinity in *Conus* (Linnaeus, 1758) shells, and Surge *et al.* (2001) found that for oysters in estuaries, $\delta^{13}C$ values can be used as a proxy for salinity. Populations of open ocean molluscs have in several instances been discerned from those of near-shore populations of the same species by their enriched $\delta^{13}C$ composition (Lécuyer *et al.* 2004, Lorrain *et al.* 2004).

No cyclic signal is recorded in the $\delta^{13}C$ data sets of the three analyzed specimens, and a correlation between $\delta^{13}C$ and $\delta^{18}O$ values is not apparent. However, all the specimens show a clear trend to lighter values in the later ontogenic stages. A similar trend can be observed in the data of Allmon *et al.* (1992, 1994) and in analyses of other turritelline species (Petsios, pers. comm. 2011, Waite and Allmon submitted).

Only adult specimens of *Turritella leucostoma* were found inhabiting the tidal flat and no live individuals smaller

than 55 mm were encountered in this study, suggesting that juveniles live in deeper water. Nothing, however, is known about the dietary preferences or biology of these juveniles. The black arrows in Figure 8 A–C mark the 55 mm length on the measured shells and, therefore, the approximate age at which the animals in question are expected to have colonized the shallow water habitat on the flat. This point roughly corresponds to the beginning of the first cold season in specimens *Tl1* and *Tl3* and the beginning of the second warm season in *Tl2*. In all three specimens it coincides with the beginning of an enhanced decreasing trend in the $\delta^{13}C$ values. The brief increase in $\delta^{13}C$ values observed in specimen *Tl3* at approximately 65 mm is associated with a predation scar and the sample corresponds to shell material that was formed during repair. The excursion is thought to be related to this feature.

At least three hypotheses might explain this observed trend towards depleted $\delta^{13}C$ values in the later ontogenetic stages of *Turritella leucostoma*:

1) A change in dietary preferences occurs from a food source with a predominantly heavy composition such as certain diatoms (~-15‰; Fry and Wainright 1991), benthic micro algae (~-16‰; Riera and Richard 1996) or certain marine plants (~-10‰; Mook and de Vries 2001) to one with a significantly lighter composition, such as terrestrial C3 plant debris (~-20‰ to -37‰; Kohn, 2010) after approximately the first year of life.

Values for respired CO_2 of -10‰ and -30‰ for heavy and light food, respectively, can be introduced into the simple model for derived shell $\delta^{13}C$ fractions expressed in equation (1) of McConnaughey and Gillikin (2008).

(1) $R \times (\delta^{13}C_{Org}) + (1 - R) \times (\delta^{13}C_{Ambient}) = (\delta^{13}C_{Blood\ DIC}) = (\delta^{13}C_{Shell}) - \Delta$

With: R = parts respired carbon; $(\delta^{13}C_{Org})$ = carbon isotopic composition of respired carbon; $(\delta^{13}C_{Ambient})$ = carbon isotope composition of ambient DIC.

When applying average values of +1‰ for ambient DIC (Mook and de Vries 2001), and Δ = +2.7‰ (Romanek *et al.* 1992, McConnaughey and Gillikin 2008) for aragonite fractionation from blood, and assuming a 10% contribution of respired carbon, then shell $\delta^{13}C$ maxima and minima equate to compositions of +2.6‰ and +0.6‰. Although these figures correspond well to the range of measured $\delta^{13}C$ shell values and demonstrate that a dramatic change in the food source could account for the observed trend, it is unlikely that the animals in question were feeding exclusively on light terrestrial organic carbon by the end of their second year of life. Furthermore the arid nature of the climate would suggest that a proportion of the terrestrial vegetation consists of drought-adapted succulent plants with obligate crassulacean acid metabolism (CAM), which have a heavier $\delta^{13}C$ signal (Mooney *et al.* 1989).

2) The ^{13}C-depleted signature of the adult stages, which live in shallow tidal flat environments relative to the juveniles, which presumably live in open marine waters, results from elevated levels of oxidation of organic matter with a low $\delta^{13}C$ composition on the tidal flat (Lécuyer *et al.* 2004; Lorrain *et al.* 2004). This effect may be enhanced by the influence of depleted pore water DIC associated with a predominantly infaunal mode of life of the adult *Turritella leucostoma*.

Near shore ambient DIC is expected to be lighter than open ocean DIC (Lécuyer *et al.* 2004; Lorrain *et al.* 2004). Furthermore, the infaunal, near-shore, filter-feeding mode of life, associated with light pore water and terrestrial derived particulate matter, has been ascertained only for the adult population, whereas nothing is known about the biology of the juveniles. Walter *et al.* (2007) found that pore waters of sediments in Florida were on average -1.8‰, almost 1.5‰ lighter than the overlying marine waters. Assuming that the $\delta^{13}C$ composition of the shell mainly reflects ambient DIC, the range of observed shell values could be explained solely by an epifaunal versus infaunal mode of life. However the change to lighter $\delta^{13}C$ seems to be gradual. This suggests that it does not reflect two discretely different environments such as pore water versus marine water, or deep water versus shallow water. However, the elevated incorporation of depleted carbon does seem to coincide with the appearance of the animals on the tidal flat.

3) Higher metabolic rates provide more light, respired CO_2 to the pool of carbon available for calcification, increasing the fraction of respired CO_2 relative to ambient CO_2 available for shell secretion. This might be related to maturity and reproduction (Allmon *et al.* 1992), predation pressure (Tull and Boehning-Gaese 1993) or a different allocation of fats versus other tissues. The effect might be especially important in the context of reduced growth rates in the later ontogenetic stages, as proposed by Allmon (2011).

Data on metabolic rates in turritellines is scarce. Fluorescein dye experiments performed on *Turritella gonostoma* have suggested relatively strong water pumping abilities. However, measured rates of filtration in adult specimens of the same species from experiments in tanks suggested relatively low metabolic rates (Allmon *et al.* 1992). More data from tracer experiments is needed to estimate metabolic rates in turritellines and evaluate the potential of increased respiratory CO_2 being incorporated into the shell in adult specimens. Nevertheless, the gradual nature of the change to lighter $\delta^{13}C$ values suggests a progressive modification of the fraction of light carbon incorporated into the shell. By applying the known shell values into formula (1) and assuming an ambient DIC composition of +1.0‰ and an average particulate organic matter composition of -22‰ (Mook and de Vries 2001), the minimum and maximum contributions of respiratory carbon equate to 4.8% and 13.5%, respectively.

The observed trend to more depleted ^{13}C values is likely to represent a signal caused by a combination of the effects discussed above. However, these effects seem to be related to—or triggered by—the migration of the adults from the deeper habitat to the shallow habitat. Unfortunately the respective contributions of these different effects are not quantifiable at present.

For six out of seven known modern species, including *Turritella gonostoma* from the Gulf of California, veligers hatch from egg masses and undergo an early planktonic larval stage lasting from 3–21 days, during which time, they can presumably be widely dispersed. This provides for a method to rapidly and efficiently remove the veligers from the tidal flat environments. Tidal flat environments with strong breakwater and tidal currents are extremely dynamic, and tiny shallow infaunal turritellines with no means of attachment would be utterly exposed to the elements if they remained in shallow water. It is hypothesized that they settle from the planktonic larval stage in deeper and calmer settings. They then "migrate" back to the tidal flat where they appear approximately after their first year of life.

The observations on habitat differences between young and mature specimens in this study can help explain narrow, right-skewed size-frequency distributions, which are observed in many fossil and modern shallow-water turritelline assemblages (Allmon 2011).

CONCLUSIONS

In conclusion, the studied tidal flat is colonized by a population of adult *Turritella leucostoma* who preferentially favor high-energy environments, which are almost constantly covered with water, such as tidal channels and the low tide beach. Oxygen isotope sclerochronology suggests that large individuals reach an age of two years and implies that the observed population consists of adults between approximately 1 and 2 years in age. The carbon isotopic composition of the analyzed shells shows a trend to lighter values for specimens larger than 55 mm. This might be related to a change in the composition of diet and/or ambient DIC associated with a change in habitat. Higher metabolic rates—possibly associated with sexual maturity and/or reduced growth rates—might account for increased availability of respiratory carbon with lighter $\delta^{13}C$ values for calcification of shells. The turritelline carbon data does not show clear cycles that could be related to seasonal changes in productivity, and as there is no covariance between oxygen and carbon data, the pattern cannot be attributed to changes in salinity related to fresh water runoff.

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Differential settlement of associated species on *Ostrea puelchana* d'Orbigny, 1842 (Ostreidae) in Patagonia (Argentina)

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Abstract: *Ostrea puelchana* d'Orbigny, 1842 is a common species of commercial interest in Patagonia and is distributed from Rio Grande do Sul (Brazil) to San Matías Gulf (SMG, Argentina). In SMG, the species develops natural banks that provide irregular surfaces suitable for colonization of organisms. We studied the composition and frequency of encrusting and associated species on *O. puelchana* shells as well as the preferential settlement of epibionts on different areas within left and right valves. A total of 55 taxa were identified. The dominant groups were Annelida, Foraminifera, Bryozoa and Mollusca in two different oyster banks. The lifestyle of the oyster favors a preferential settlement of epibionts on different valves and areas within the valves. Substratum heterogeneity, reproductive cues, gregarious behavior, protection against predation and/or brooding care could be responsible for this differential settlement. The left valve was more encrusted than the right one. Spirorbinae, Cirratulidae, Foraminifera, juvenile *O. puelchana*, Bryozoa and Hydrozoa showed preferential settlement in different areas on the external left valves. On the external right valves, the same taxa except for Hydrozoa showed a nonrandom distribution between areas.

Key words: Epibiosis, oyster, preferential colonization, SW Atlantic Ocean

Colonization of hard substrates is a well-known phenomenon in marine environments since the Archaean (Taylor and Wilson 2003). In such environments, availability of stable substrate for the settlement of sessile organisms is a main resource in the colonization process (Wahl 1989). However, in soft bottoms, a wide variety of biogenic, abiogenic and even anthropogenic surfaces are often occupied and provide available substrate for settlement and shelter of benthic invertebrates (Taylor and Wilson 2003).

Epibiosis is defined as a spatial and non-symbiotic association in which a living organism is used as substrate (basibiont) by a sessile organism (epibiont). Trophic exchange with the substrate organism, if present, is facultative (Wahl 1989). Usually, this interaction is not specific (Barnes and Clarke 1995, Cook *et al.* 1998, Wahl and Mark 1999, Williams and McDermott 2004) and both organisms may experience advantages and disadvantages depending on the species involved in the association and on the environmental variables (Wahl 2009). In consequence, the nature of the effects of epibionts on basibionts is often context-specific (Hay *et al.* 2004, Wahl 2008).

Oysters are ubiquitous and provide stable substrates for settlement and development of benthic communities (Dauer *et al.* 1982, Zimmerman *et al.* 1989, Rosell *et al.* 1999). In soft bottoms, oyster larvae or spat settle on adult oysters, shell fragments, rhizomes and stems of *Spartina* Schreb or consolidated sediment (M.V. Romero pers. obs., Escapa *et al.* 2004, Borges 2006, Kochmann *et al.* 2008). Thus, they generate aggregates or clusters on adult oysters resulting in large accumulations that can modify the environment with their own physical structure and create different habitats available for colonization (Gristina *et al.* 1996, Barnes 2001, Zuschin and Baal 2007).

Oyster reefs and associated fauna also play an important role in carbon, nitrogen and phosphorus cycles, allowing the mineralization of organic carbon and the release of nitrogen and phosphorus available to primary producers (Parras and Casadío 2006). They can modify the speed and turbulence of water flow as a consequence of growth in aggregates, causing changes in the availability of resources that affect other organisms (Lenihan 1999). Finally, reefs may offer protection against predation and physical stress generated by wave action (Kochmann *et al.* 2008). These changes in structure and performance of an ecosystem caused by certain species have been included in the conceptual framework of ecosystem engineering (see Jones *et al.* 1994, 1997).

Numerous recent papers have focused on key issues such as distribution, diversity, management, conservation and restoration of oyster reefs and coastal marine estuarine systems throughout the world (7th International Conference on Shellfish Restoration 2005, Carranza *et al.* 2009). Most knowledge available about changes in diversity of benthic communities associated with oysters compare the macrofauna composition

between exploited and unexploited reefs or between reefs and surrounding habitats or substrates (de Grave *et al.* 1998, Escapa *et al.* 2004, Hosack *et al.* 2006, Rodney and Paynter 2006, Markert *et al.* 2010, Lejart and Hily 2011). However, few studies have examined the shell surfaces of oysters and associated epifauna collected from natural reefs, banks or beds.

Ostrea puelchana d'Orbigny, 1842, often known as "puelche" or "Patagonian oyster", is a common species in Patagonia that belongs to the family Ostreidae, subfamily Ostreinae (Stenzel, 1971). The shell is inequivalve; right valve tends to be flat and smooth whereas the left valve is convex and rough with pronounced ribs and scarce lamellae. The right valve is covered by many conchiolinous growth squamae (*i.e.*, lamellae) that develop from the center of valve toward the labrum, reaching thicknesses greater than 1.5 cm.

Ostrea puelchana is an endemic/native species (Sacco, 1897) widely distributed from Rio Grande do Sul (27°–35°S, Brazil) to San Matías Gulf (SMG) (40°–42°S, Argentina), where major banks with commercial interest are reported (Castellanos 1957, Rios 1970). Recently, Oehrens Kissner *et al.* (2011) recognized the development of small banks in San José Gulf (42°20′S, 64°20′W). *Ostrea puelchana* can reach a commercial height of 120 mm, although fishery is not established in the market (Borges 2006). The species develops natural extensive banks that provide highly irregular surfaces suitable for colonization of other organisms. However, information about the biota associated with its shells is scarce and refers to particular infestations (Mauna 2003, Cremonte *et al.* 2005, Rodríguez 2007, Diez *et al.* 2011). We studied the composition, frequency and distribution of encrusting and associated species on the shells of *O. puelchana* in SMG. The preferential settlement of epibionts on different areas within left and right valves was evaluated.

MATERIALS AND METHODS

Study area

SMG is located between 40°42′–42°41′S and 63°45′–65°09′W and presents important biological and fishery production (Morsan 2002, Narvarte *et al.* 2011). This is a semi-enclosed area within the Argentinian shelf with particular oceanographic features (Guerrero and Piola 1997), maximum depths near 200 m in the central area (Parker *et al.* 1997) and a macrotidal regime (Servicio de Hidrografía Naval 2010). The average salinity is high (33.84) and the average annual temperature is 13.25 ± 0.20 °C with strong thermal stratification mainly in summer (Rivas 1990). The bottom type is dominated by sands with high contents of silt and clay (Parker *et al.* 1997). Oyster banks are located on sand or sand-gravel facies deeper than 10 m depth (Escofet *et al.* 1978).

Sampling

Oysters ($N = 142$) were collected in February 2009 in two natural banks located at northwest of SMG (Fig. 1), called *El Buque* (EB, 40°50′S, 65°10′W) and *Zona de Colectores* (ZC, 40°56′S, 65°06′W), at 12 and 18 m depth at low tide, respectively. Samples were taken randomly within the most densely packed zone of each bank. Oysters were placed in tanks with circulating sea water. To avoid loss of macrofauna associated to the valve, each oyster was stored individually in a plastic bag. Oyster samples were fixed in 5% seawater formalin and 15 days later they were stored in 70% alcohol.

The epibionts and associated fauna of each left/right valve and internal/external shell surface were identified. Both surfaces were mainly considered because an edge along the internal left valve was often available to organism settlement due to breakage of young lamellae of right valves.

Areas were defined on each valve to test preferential colonization. Zonification maps of both valves were used in order to recognize the frequency and distribution of encrusting taxa. This map reflects dissimilar morphological features of the valves that may influence the settlement of different marine larvae and could be used to identify those areas where epibionts may affect the development of the oyster. The external left valve was divided into six areas: apex (10%), platform (5%), anterior margin (15%), ventral margin (25%), posterior margin (15%) and center (30%). There is no platform in the right valves, so the areas were: apex (10%), anterior margin (15%), ventral margin (30%), posterior margin (15%) and center (30%). Presence/absence, abundance (*i.e.*, number of individuals) and/or coverage (*i.e.*, percentage of the area encrusted) data were recorded in standardized maps of each valve (Fig. 2). In the internal left valve, the total available surface to be colonized by epibionts was attributed to edge of

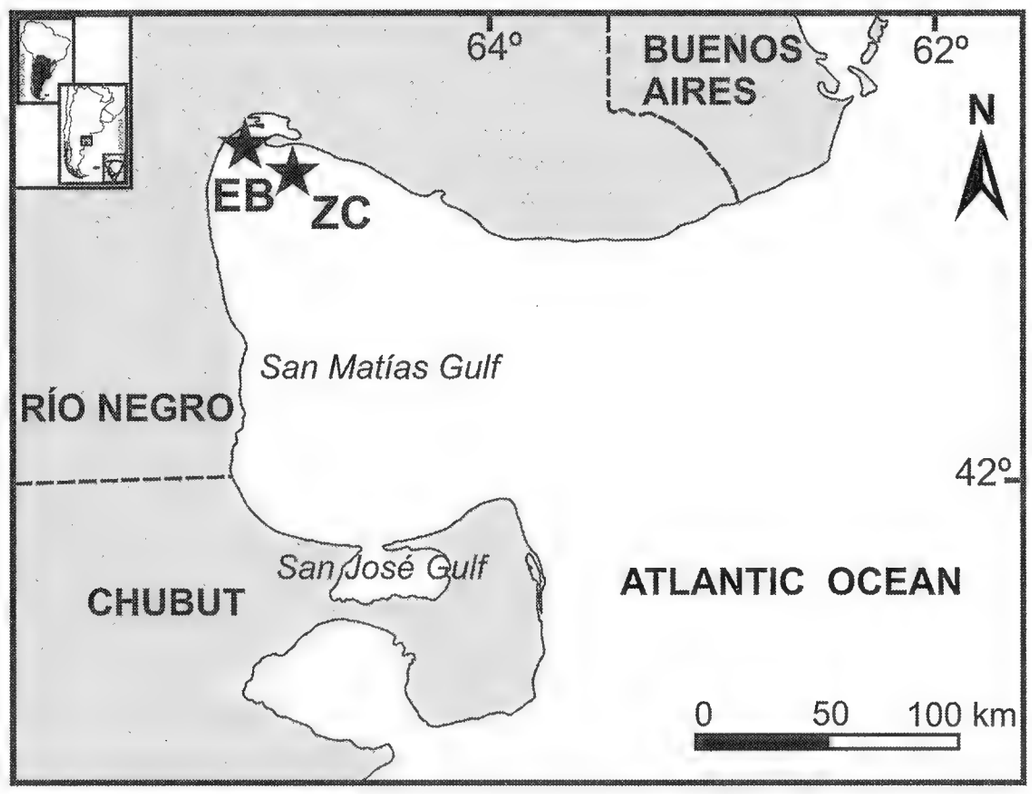

Figure 1. Location of the two oyster banks (**EB** and **ZC**) in San Matías Gulf (SMG, Argentina).

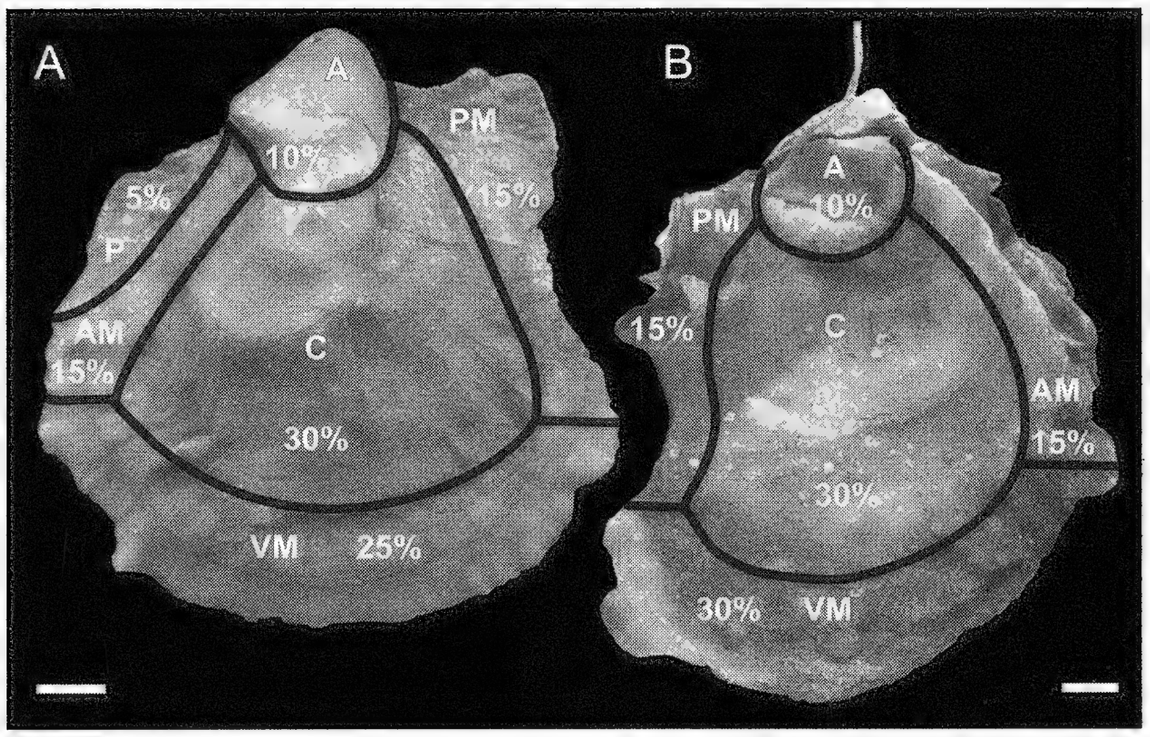

Figure 2. Zonification maps with areas selected in left valves (**A**) and right valves (**B**) of *Ostrea puelchana* (external surface). **A,** apex, **AM,** anterior margin, **C,** center, **P,** platform, **PM,** posterior margin, **VM,** ventral margin. Coverage percentage is indicated in each area. Scale bars = 1 cm.

valve. This surface was divided into five areas: apex (15%), platform (15%), anterior margin (20%), ventral margin (30%) and posterior margin (20%). In the internal right valve, the preferential settlement was not evaluated due to the very low observed frequencies.

The percentages were assigned arbitrarily and were estimated for each taxon, following Ward and Thorpe (1991) and Mauna *et al.* (2005). Coverage data were registered for those taxa with colonial forms and for epibionts that can be counted as individuals but can occupy large areas of the valves.

Data analysis

Richness of associated fauna and epibiota on the oyster shells were compared using Multivariate Nonparametric Analysis (PRIMER 6.1.10, see Clarke 1993, Clarke and Warwick 2001). Multidimensional Scaling (MDS), Similarity Percentage (SIMPER) and Analysis of Similarities (ANOSIM) tests were applied to make comparisons between EB and ZC banks (presence/absence data, Jaccard similarity index). MDS and SIMPER analysis were applied to compare right/left valves and their external/internal surfaces using abundance (fourth root transformed data, Bray-Curtis index) and/or coverage (percentage data, Bray-Curtis index). The arithmetic mean of abundances was calculated to identify if right or left valves and external or internal surfaces were more frequently colonized.

Goodness of fit test and exact confidence intervals for the binomial distribution were performed in order to assess possible preference of epibionts on different areas of the valves. When more than 20% of the expected frequencies were less than five, valve areas were grouped to avoid inaccuracies. Yates's correction for continuity was applied in those cases with only one degree of freedom, and relatively small samples (Zar 1999). The null hypothesis was that the distribution of epibionts on valves is at random on the significance level $\alpha = 0.05$.

RESULTS

Epibionts were present in all the oyster specimens (N = 142) collected in EB and ZC banks. A total of 55 taxa belonging to 12 taxonomic groups were identified (Table 1). The highest percentage of occurrence in both banks (EB = 96.5% and ZC = 77.9%) corresponds to polychaetes of the family Cirratulidae, represented by 11 taxa. Other epibionts, including associated fauna, boring and encrusting organisms (*i.e.*, ctenostome bryozoans, skeletons of calcareous algae and barnacle basal plates) occurred only occasionally. Gnawing traces possibly made by the radulae of chitons on the external surfaces of both valves were recognized, most frequently in left (EB = 91% and ZC = 87%) than right valves (EB = 46% and ZC = 35%).

The MDS plot showed a clustering of samples from EB and ZC banks (stress = 0.19) and ANOSIM test indicated no difference in community composition (global $R = 0.099$, P = 0.1). SIMPER procedure (Table 2) revealed that Spirorbinae, Bryozoa, Cirratulidae and *Ostrea puelchana* were the taxa that contributed most to the average similarity within every bank (> 62%).

In EB and ZC, MDS between external and internal surfaces of both oyster valves showed two groupings of samples with abundance (stress = 0.12) and coverage (stress = 0.11) data. SIMPER results (Table 2) showed high percentages of average dissimilarity between external and internal surfaces ($\overline{\delta}_{\text{coverage}} = 88.02\%$, $\overline{\delta}_{\text{abundance data}} = 79.18\%$), being Spirorbinae, *Ostrea puelchana*, Bryozoa, Cirratulidae, Hydrozoa, Foraminifera and byssate mytilids the main taxa that contributed to differences. The external surface was more colonized than the internal one (Fig. 3).

Regarding epibiosis in left and right valves, MDS plots based on coverage (stress = 0.15) and abundance (stress = 0.13) data indicated a weak grouping of samples. SIMPER results (Table 2) showed average dissimilarities higher than 63 % between left and right valves of *Ostrea puelchana* ($\overline{\delta}_{\text{coverage}} = 63.37\%$, $\overline{\delta}_{\text{abundance data}} = 64.70\%$). The taxa that most contributed to the differences were Cirratulidae, Spirorbinae, Foraminifera and *O. puelchana*. The left valve was, in general, more colonized than the right one (Fig. 4).

The preferential settlement of epibionts on different areas of left and right valves is shown in Figs. 5 and 6, respectively. On the left valve, Spirorbinae preferably colonized the platform and the center of the external surface; while the

Table 1. List of epibionts and associated taxa found on *Ostrea puelchana* sampled at EB and ZC (SMG, Argentina).

Taxa
Algae
calcareous skeletons
Foraminifera
Miliolinella subrotunda (Montagu, 1803)
Quinqueloculina d'Orbigny, 1826
Quinqueloculina lamarckiana d'Orbigny, 1839
Quinqueloculina angulata (Williamson, 1858)
Discorbina valvulata (d'Orbigny, 1839)
Bolivina doniezi Cushman and Wickenden, 1929
Cibicides fletcheri Galloway and Wissler, 1927
Lobatula lobatula (Walker and Jacob, 1798)
Discorbis Lamarck, 1804
Planorbulina variabilis (d'Orbigny, 1826)
Trochammina Parker and Jones, 1859
Pyrgo Defrance, 1824
Pyrgo ringens (Lamarck, 1804)
Porifera
Cliona celata Grant, 1826
Cnidaria
Anthozoa unidentified
Hydrozoa (hydrocauli)
Nematoda
Nematoda unidentified
Annelida
Serpulinae tubes
Spirorbinae
Phyllochaetopterus Grube, 1863
Lumbrineridae
Eunice argentinensis (Treadwell, 1929)
Phyllodocidae
Syllidae
Maldanidae
Spionidae
Monticellina Laubier, 1961
Caulleriella Chamberlin, 1919
Tharyx Webster and Benedict, 1887
Cirratulus Lamarck, 1801
Cirratulus sp. 1
Cirratulus sp. 2
Cirratulidae unidentified 1
Cirratulidae unidentified 2
Cirriformia Hartman, 1936
Chaetozone Malmgren, 1867
Aphelochaeta Blake, 1991
Cirratulidae (multitentaculate)
Sipunculida
Sipunculida unidentified
Brachiopoda
Juvenile Brachiopoda
Mollusca
Ostrea puelchana d'Orbigny, 1842
Crepidula Lamarck, 1799
Leiosolenus patagonicus (d'Orbigny, 1842)
Mytilus edulis Linnaeus, 1758
Aulacomya atra (Molina, 1782)
Chaetopleura Shuttleworth, 1853
Arthropoda
Cirripedia (basal plates)
Bryozoa
Escharoides Milne Edwards, 1836
Escharoides sp. 1
Escharoides sp. 2
Microeciella Taylor and Sequeiros, 1982
Copidozoum Harmer, 1926
Cyclostomatida unidentified
Ctenostomatida unidentified
Bugula Oken, 1815
Chordata
Ascidiacea unidentified

more settled areas of the internal surface were the platform and the posterior margin. Cirratulidae were found on the external surface, preferentially on the ventral margin. *Ostrea puelchana* recruits occurred preferentially on the platform and the anterior margin of the external left valve and, on the internal one, their frequency was significantly higher than expected only on the platform. Furthermore, the platform was preferentially colonized by Bryozoa in both surfaces and only the posterior margin on the internal left valve. Hydrozoa showed preferential location in the platform, apex and margins on the external left valve and only in platform and apex on internal left valve. On the external surface of left valves Foraminifera preferentially settled on the apex, platform and center. On the internal surface of left valves, observed frequencies of *Phyllochaetopterus* Grube, 1863 were significantly higher than expected on the platform and apex. In the external right valve, Spirorbinae, Foraminifera, Cirratulidae, *O. puelchana* recruits and Bryozoa showed a nonrandom distribution between areas. Spirorbinae encrusted preferentially the apex and center,

Foraminifera bored on the apex and Cirratulidae were preferentially distributed on the margins. *Ostrea puelchana* recruits showed a preferential settlement on the center. Particularly, Bryozoa showed differential settlement only on the ventral margin with observed frequencies lower than the expected ones.

DISCUSSION

In this study, all the specimens of *Ostrea puelchana* examined showed epibionts. Oysters were associated with 55 taxa of sedentary and free-living organisms recorded on both valves. The dominant groups were Annelida (20 taxa), Foraminifera

Table 2. Dissimilarity percentages between banks (EB/ZC), kind of surfaces (external/internal) and valves (left/right). Contributions percentages of each taxa higher than 3% were considered.

	DISSIMILARITY PERCENTAGES				
	EB vs. ZC	External vs. Internal Surface		Left vs. Right Valves	
Taxa	Presence/Absence	Coverage	Abundance (transformed data)	Coverage	Abundance (transformed data)
Spirorbinae	-	37.69	34.12	19.85	26.78
Cirratulidae	6.3	-	26.65	-	30.25
Ostrea puelchana	8.58	21.55	14.88	26.62	14.65
Foraminifera	11.73	-	11.62	-	13.4
Bryozoa	9.87	20.61	-	24.85	-
Hydrozoa (hydrocauli)	9.08	5.42	-	6.34	-
byssate mytilids	8.68	5.18	-	8.36	-
Phyllochaetopterus sp.	8.41	-	-	4.59	-
Sipunculida unid.	5.6	-	-	-	-
Crepidula sp.	4.64	-	-	-	-
Cliona celata	4.32	-	-	-	-
Lumbrineridae	3.34	-	-	-	-
Leiosolenus patagonicus	3.3	-	-	-	-

(13 taxa), Bryozoa (7 taxa) and Mollusca (6 taxa) in both oyster banks. Within a local biodiversity framework, the present inventory largely extends the knowledge about benthic species richness in SMG. Previous records of macro and micro-fauna associated with *O. puelchana* in natural habitats include a few mollusks (*Mytilus platensis* d'Orbigny, 1842, *Aulacomya atra* (Molina, 1782) and Calyptraeidae), together with polychaetes (*Spirorbis* Daudin, 1800), crustaceans (*Balanus* Costa, 1778), echinoderms, epizoic bryozoans, ascidians and foliculinid Protozoa (Castellanos 1957). In San José Gulf, seven similar taxa colonized *O. puelchana* but in very low percentages (Cremonte *et al.* 2005). In a study of the role of chitons in the settlement of *O. puelchana* recruits on conspecific adults, Pascual (1997) reported that ascidians were the most conspicuous epibionts in the absence of grazers at natural oyster banks in SMG. Regarding composition and frequency of epibionts on oyster shells, other studies worldwide give similar results and show the presence of similar epibiotic and endolithic organisms in different marine environment from all geographic regions (Barnes 2001, Guenther *et al.* 2006, Smyth and Roberts 2010).

Boring organisms and bioerosional structures produced by boring activity upon the shells were also recorded on valves of *Ostrea puelchana*. Bioerosion traces attributed to Porifera—*e.g.*, *Cliona celata* Grant, 1826, Foraminifera, Bryozoa Ctenostomatida, Polychaeta Spionidae and Bivalvia, *e.g.*, *Leiosolenus patagonicus* (d'Orbigny, 1842)—as well as byssal etchings produced by the anchoring of bivalves to the substrate and parallel sets of straight to curved scrape marks representing gnawing traces attributed to radulae of chitons were recognized in this study. Due to the commercial value of *O. puelchana*, few studies aim to investigate the infestation produced by *Polydora rickettsi* Woodwick, 1961, *C. celata* and *L. patagonicus*

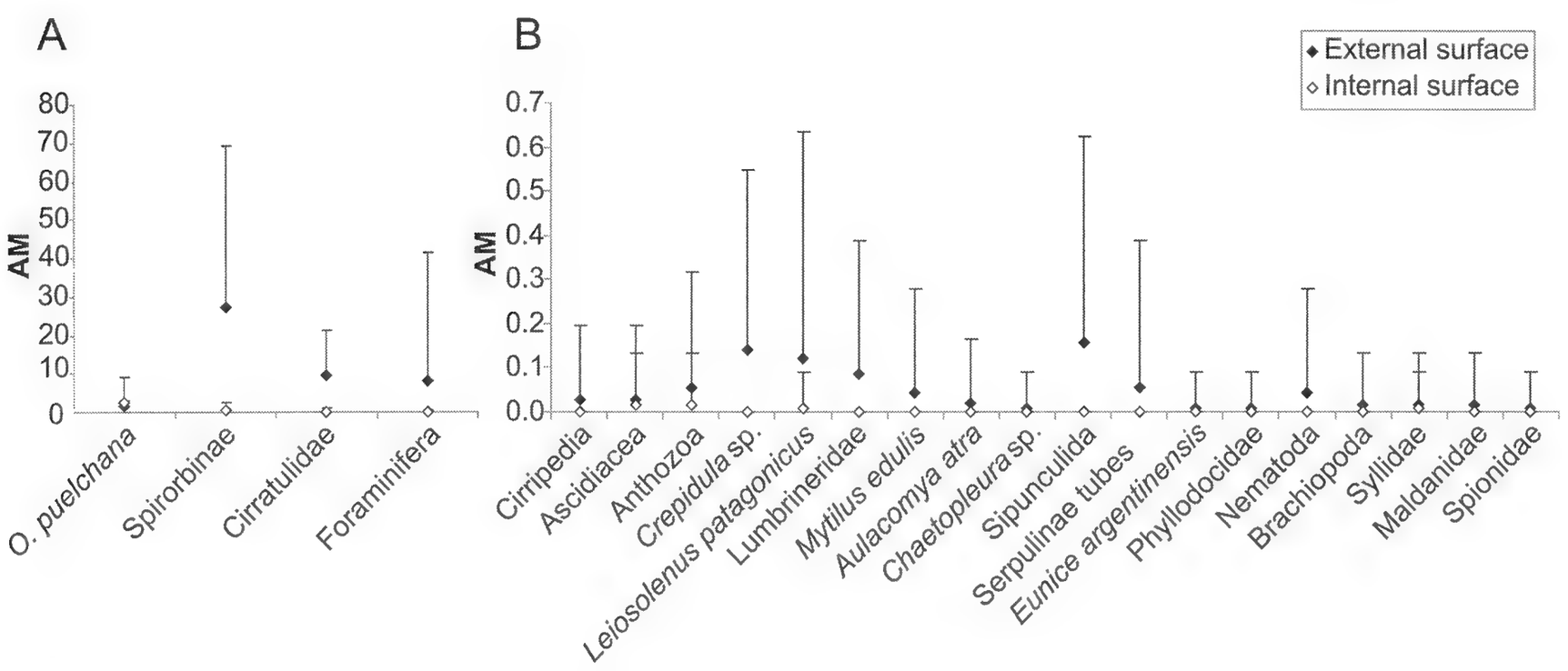

Figure 3. Arithmetic mean (AM) of abundance of taxa registered on the external and internal surface. **A,** More frequent taxa and **B**) uncommon taxa. The error bars indicate the standard deviation of the data.

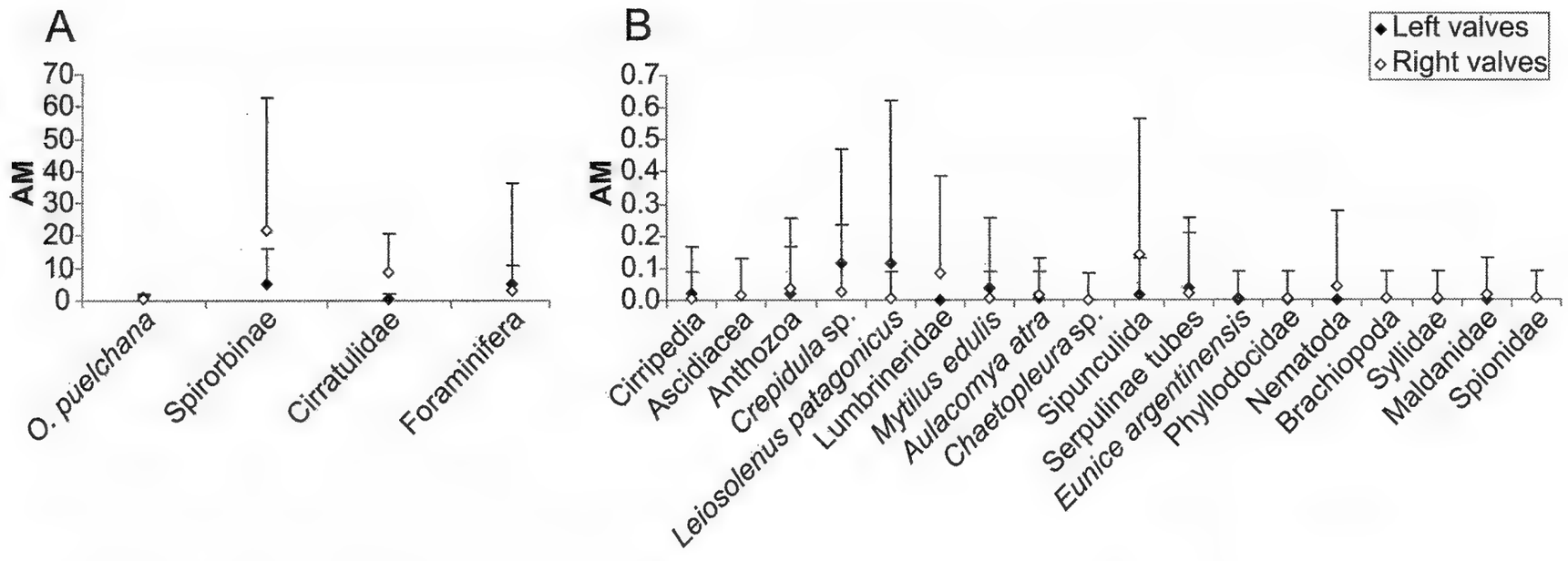

Figure 4. Arithmetic mean (AM) of abundance of taxa registered on left and right valves. **A)** More frequent taxa and **B)** uncommon taxa. The error bars indicate the standard deviation of the data.

(Mauna 2003, Cremonte *et al.* 2005, Rodríguez 2007, Diez *et al.* 2011). These taxa were frequently reported in different species of oysters (Doroudi 1996, Wesche *et al.* 1997, da Silva *et al.* 2010, Sabry *et al.* 2011).

Marine organisms that colonize both biogenic and abiogenic hard substrates often exhibit a pattern of uneven distribution between exposed and less exposed or cryptic surfaces (Palmer and Fürsich 1974, Ward and Thorpe 1991, Nebelsick *et al.* 1997, Glasby and Connell 2001, Schejter and Bremec 2007a, 2007b). On the "puelche oysters", the external surface of valves supported a high coverage of epibionts. Particularly, recruits of *Ostrea puelchana* showed greater percentage of coverage on external than on internal surfaces, although higher numbers were recorded on the internal surface, mainly in the platform and anterior margin of left valves. Considering that the oysters were collected in breeding season, the preferential settling of juveniles probably reflects the carriage of dwarf males by adult females, as described only in *O. puelchana* (Calvo and Morriconi 1978, Pascual 1997). The non-random distribution of these juveniles was attributed to some kind of chemical interaction female-epibionts (Calvo and Morriconi 1978, Pascual and Zampatti 1995). Besides, Pascual (1997) proposed that this non-random distribution, at least in part, is a consequence of the increased survival rate of epibionts settled on the platform of adult females, which operates as a refuge from grazing by chitons. In this study, although chitons were occasionally found, gnawing traces were registered at high frequencies, mostly on the external surfaces of left shells. Left valves of *O. puelchana* exhibited a greater coverage and number of epibionts than right valves. Nevertheless, some taxa (Anthozoa, Nematoda, Sipunculida, Bryozoa, Polychaeta Spirorbinae and Cirratulidae) were most conspicuous on right valves, most of them in the shell margins and associated to lamellae. Rosso and Sanfilippo (1991) report similar results in the scallop *Zygochlamys patagonica* (King and Broderip, 1832) from the Beagle Channel, with increased coverage of epibionts on the left valves and a greater colonization of epibionts and associated fauna on the margins of right valves, without contact with the substrate. In contrast, Smyth and Roberts (2010) reported similar degree of epibiosis in both valves of *Ostrea edulis* Linnaeus, 1758, probably because the oysters settled at nearly 45° on the bottom. The life position of *O. puelchana* provides stability in a highly hydrodynamic environment as it lies with the right valve in contact with the substrate (Pascual 1993). The lifestyle of the "puelche oyster" favors a preferential settlement of epibionts on different valves and areas within the valves.

Additionally, the texture of a colonized surface is an important factor that can influence the settlement of larvae of benthic invertebrates (Eckman 1990, Hoover and Purcell 2009). The preferential settlement in cryptic habitats is common to most marine invertebrate larvae and may primarily be an evolutionary adaptation to prevent mortality by solar radiation, and secondly, to avoid mortality by sedimentation and predation (Svane and Dolmer 1995). Rough and irregular surfaces are more attractive than smooth surfaces for the settlement of organisms (Warner 1997). This would explain the greater coverage and number of epibionts, not susceptible to grazing, on the rough left valves than on the smooth right valves. Small scale substratum heterogeneity (*e.g.*, 1 mm) affects the larval settlement and subsequent development of epibenthic community (Lapointe and Bourget 1999) and habitats of great complexity will increase the biodiversity of the assemblages that occur within them (Huston 1997, Tilman *et al.* 1997, Tilman 1999). The imbricate concentric lamellae on right valves of *Ostrea puelchana* seem to be a good example at individual scale. Lamellae form a fringe around the margin of the shell and generate microhabitats available for colonization. These microhabitats harbored 11 taxa of Cirratulidae and others less abundant. In accordance with these results, Kalyanasundaram *et al.* (1974) found that protected areas and cavities on valves of Ostreidae were habitats of *Cirratulus cirratus* (Müller, 1776). Liñero-Arana and Diaz (2006) also indicated the presence of polychaetes Cirratulidae and Sabellidae on the mollusk *Spondylus americanus* Hermann, 1781, which build their galleries with the sediment accumulated between the spines of the mollusk. Similarly, lamellae developed in right valves seem to be used as habitats that give refuge from predators and brooding care. Brooding care behavior, like the deposition of eggs in mucus on rocks and shells, is a usual strategy in Cirratulidae (Petersen 1999).

The gregarious behavior of settled organisms on marine hard substrates is a common phenomenon. Aggregation is a pattern that shows a variety of different processes,

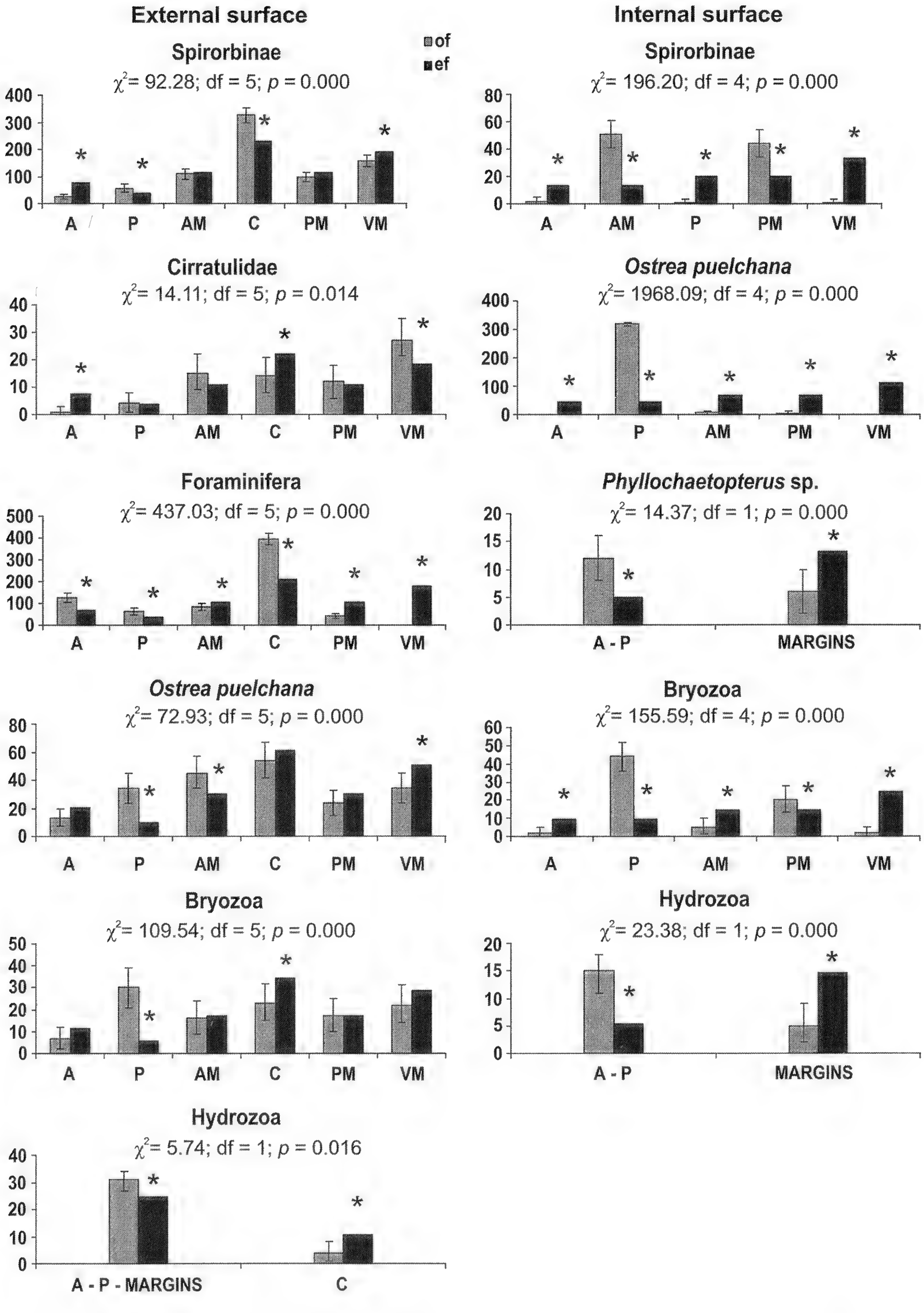

Figure 5. Preferential settlement of epibionts on different areas within external and internal surfaces of left valves. Asterisks indicate significant differences between expected frequencies (**ef**) and observed frequencies (**of**) for each area. **A,** apex, **AM,** anterior margin, **C,** center, **MARGINS,** includes anterior margin, posterior margin and ventral margin grouped, **P,** platform, **PM,** posterior margin, **VM**, ventral margin.

including differential early mortality of those individuals that settle more distantly from others, variations in the topography of the substrate surface which attract larvae, limited substrate availability and active larval selection of sites close to adults of the same species (Taylor and Wilson, 2003). Oyster larvae exhibit a gregarious behavior in response to water-soluble signals produced by conspecific adults and congeners (Hidu *et al.* 1978, Tamburri *et al.* 2008). Also, Bryozoa larvae (Wendt and Woollacott 1999, McKinney and McKinney 2002) and Serpulidae larvae (Knight-Jones 1951, James and Underwood 1994) settle on shadowy areas and show a gregarious behavior. Moreover, protection against predation and turbulence is one of the reasons for the endolithic behavior of Foraminifera (Vénec-Peyré 1996, Bromley and Heinberg 2006), drilling preferably flat areas on shells of gastropods (Smith 1988). These arguments could explain the preferential colonization on the oyster right valves.

Most of the taxa associated with *Ostrea puelchana* were selective suspension and deposit feeders. Selective deposit feeders were mainly associated with the ventral margin (*e.g.*, Cirratulidae), while selective suspension feeders (*e.g.*, Bryozoa, Spirorbinae and *O. puelchana* recruits) were preferably distributed on the dorsal areas of both valves. This differential colonization of functional groups in the areas of the valves supports the assumption that ventral margins are frequently covered by sediment.

In conclusion, this study extends the knowledge about benthic species richness at a local scale and shows that *Ostrea puelchana* possesses biogenic engineering qualities. In soft bottom environments, subjected to current action and resuspension of sediments, the substrate and microhabitats provided by *O. puelchana* increase species richness and allow the establishment and protection of mobile small individuals. Epibionts include boring and encrusting organisms, and together with other associated taxa, show a variety of living habits and trophic guilds. The lifestyle and substratum heterogeneity (*i.e.*, rough/smooth surfaces and lamellae) of "puelche oyster" favor a preferential settlement of epibionts on different valves and areas within the valves.

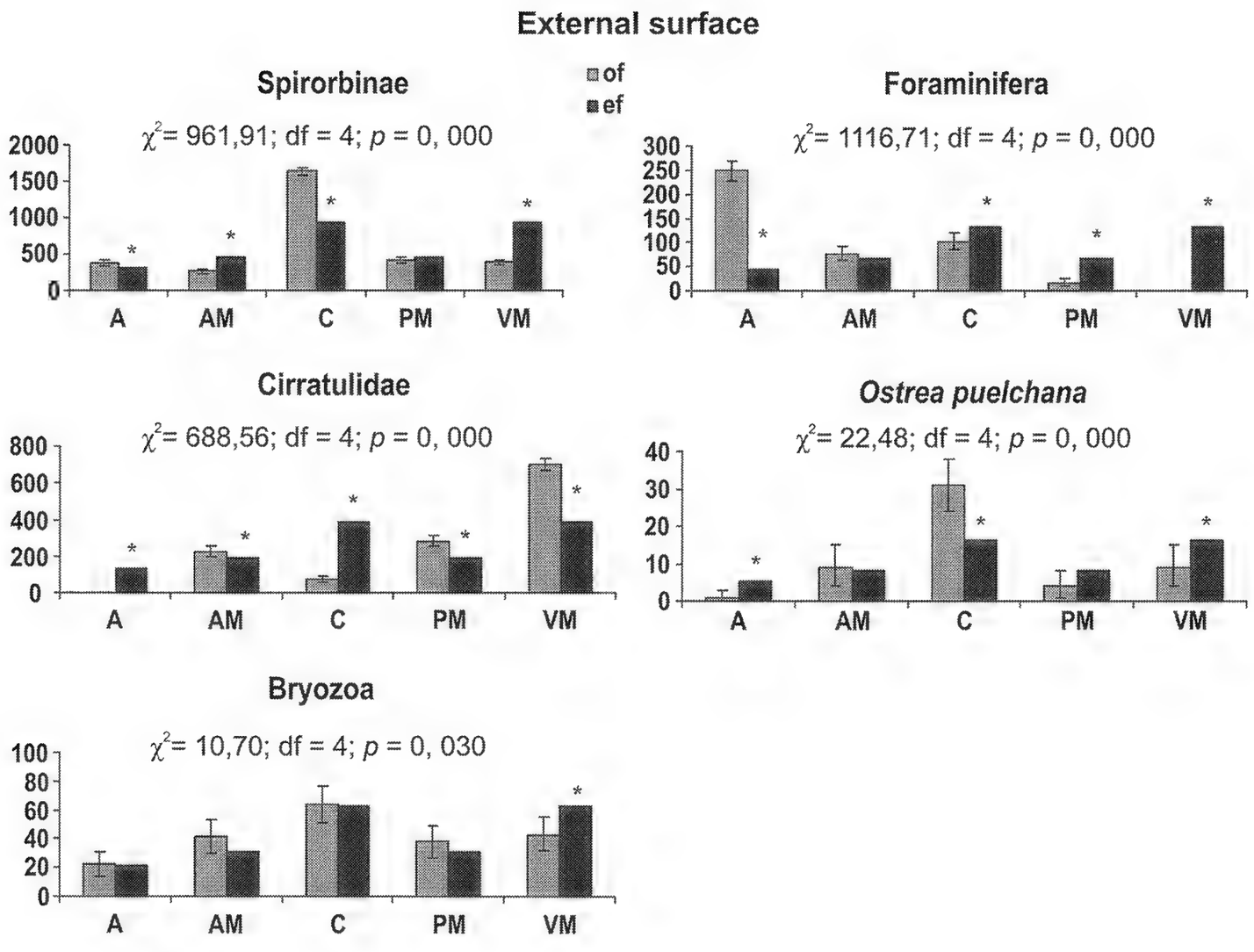

Figure 6. Preferential settlement of epibionts on different areas within external surfaces of right valves. Asterisks indicate significant differences between expected frequencies (**ef**) and observed frequencies (**of**) for each area. **A,** apex, **AM,** anterior margin, **C,** center, **P,** platform, **PM,** posterior margin, **VM**, ventral margin.

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RESEARCH NOTE

Refugial populations of *Vertigo lilljeborgi* and *V. genesii* (Vertiginidae): New isolated occurrences in central Europe, ecology and distribution

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Abstract: During the research of fen mollusc assemblages in Switzerland carried out in August 2012, we recorded the first occurrence of *Vertigo lilljeborgi* (Westerlund, 1871) in the Alps and several new occurrences of *V. genesii* (Gredler, 1856), substantially extending its Alpine distribution island towards the western part of the mountain range. Both these Euro-Asian species are mainly distributed in northern Europe and have a strong ecological affinity to groundwater-fed fens. With respect to the rarity, refugial character and high conservation value of central European populations of these species, we review in detail their ecological preferences and present European distribution, and also comment on the possible conservation implications for their globally threatened habitats.

Key words: habitat specialists, glacial relicts, fens, Switzerland

Broad palaeoecological evidence indicates that many cold-adapted species were more widespread in central Europe during the Quaternary glaciations than they are today (*e.g.*, Godwin 1949, Reisch *et al.* 2003, Dalén *et al.* 2007). The present distribution ranges of these species are often restricted to disjunct areas in northern Europe and in isolated refugia at lower latitudes (Stewart *et al.* 2010). Although these southerly located refugia are usually found in mountainous regions, they can also be represented by small island-like habitats of favorable microclimate, not necessarily situated at higher altitudes, *i.e.*, algific talus slopes (Nekola 1999).

Mire ecosystems, such as groundwater-fed fens, belong to typical refugial habitats, which are capable of sustaining cooler and more humid microclimatic conditions compared to the surrounding landscape matrix (Hampe and Jump 2011). Such unique habitat features of fens are evidenced by their exceptional biodiversity and occurrence of highly endangered habitat specialists, mostly considered to be glacial relict species (*e.g.*, Wassen *et al.* 2005, Horsák and Cernohorsky 2008, Hájek *et al.* 2011). Nonetheless, fens belong to the most seriously threatened ecosystems of the temperate zone (*e.g.*, Joosten and Clarke 2002, van Diggelen *et al.* 2006). Most likely because of the fragmentary character, rarity and isolation of mire habitats, there is a considerable lack of faunistic knowledge regarding many taxonomic groups of organisms living in fens. However, recent studies have revealed that fens harbor highly diverse mollusc assemblages of unique species composition (*e.g.*, Horsák and Hájek 2003, Horsák and Cernohorsky 2008, Horsák *et al.* 2011).

Several land snail species with high affinity to fen habitats, *e.g.*, *Vertigo genesii* (Gredler, 1856), *V. geyeri* Lindholm, 1925 and *V. lilljeborgi* (Westerlund, 1871), were widely distributed in central Europe during the Late Glacial and Early Holocene, as suggested by the fossil record (*e.g.*, Jaeckel 1962, Ložek 1964). Thus, they are traditionally referred to as relicts from these periods in temperate Europe (*e.g.*, Coles and Colville 1980, Ložek 1992, Hausdorf and Hennig 2003, Horsák *et al.* 2010). Nowadays, the genus *Vertigo* O. F. Müller, 1774 reaches its highest diversity in North America (Nekola and Coles 2010) and in Europe it is represented by 15 extant species (von Proschwitz 2003). The distribution of several of them is still poorly known, mainly due to their minute size, difficult identification and often high affinity to rare habitat types (*e.g.*, Nekola and Coles 2010). In the light of the given information, the main aims of our study were to: (1) present the first record of *V. lilljeborgi* and new records of *V. genesii* in the Alps to expand our knowledge on the distribution of these Euro-Asian land snails restricted to fens; and (2) to review the ecology and present European distribution of the target species, with possible implications for conservation of their habitats.

In the course of research of fen mollusc assemblages in Switzerland (August 2012) altogether 30 fen sites were sampled between 45°59′–47°31′N and 06°48′–10°21′E, mainly situated in the Swiss Alpine region. In the central part of each site, a sampling plot of 16 m^2 was defined and one sample of 12-liter volume was collected. Snails were extracted from samples using the 'wet sieving technique' in the field, as described

in detail by Horsák (2003). Water conductivity and water pH were measured in central parts of the sampling plots, using portable instruments with automatic temperature condensation (WTW Multi 340i/SET). Vegetation composition of both vascular plants and bryophytes was recorded in the same plots.

During the research several new isolated occurrences of *Vertigo lilljeborgi* and *V. genesii* were discovered (Fig. 1), documenting an important range extension of these species in central Europe. The site of *V. lilljeborgi* was situated ca. 193 km from its nearest known site in Schluchsee, Germany (Gerber 1987); *V. genesii* was found at few fen sites situated ca. 166 km from its nearest known site in the eastern Alps (http://lepus.unine.ch/carto). Voucher specimens are deposited in the authors' collection (Brno, Czech Republic).

We consider these newly found populations to be glacial relict, *i.e.* located outside the species' main distributional ranges in northern Europe and persistent at the sites since the Late Glacial or Early Holocene. However, direct fossil evidence to support this presumption is rather scarce, especially for *Vertigo lilljeborgi* which inhabits mineral-poor sites, generally unsuitable for shell preservation. Therefore, an alternative scenario of more recent colonization of the sites via long-distance passive dispersal on migrating birds (*e.g.*, Gittenberger *et al.* 2006) can also explain the observed distribution pattern. Indeed, such dispersal appears to be very effective for small-sized snail taxa, which, therefore, tend to exhibit continental ranges of distribution (Nekola *et al.* 2009). Nevertheless, it is rather unlikely for species confined to rare and island-like habitats to spread effectively by passive dispersal (Horsák *et al.* 2007, 2012). Therefore, the former scenario seems to be more plausible, with respect to the close linkage of studied species to relict habitats and also known Holocene continuity of some fens nearby the new site of *V. lilljeborgi* (Ilyashuk *et al.* 2009).

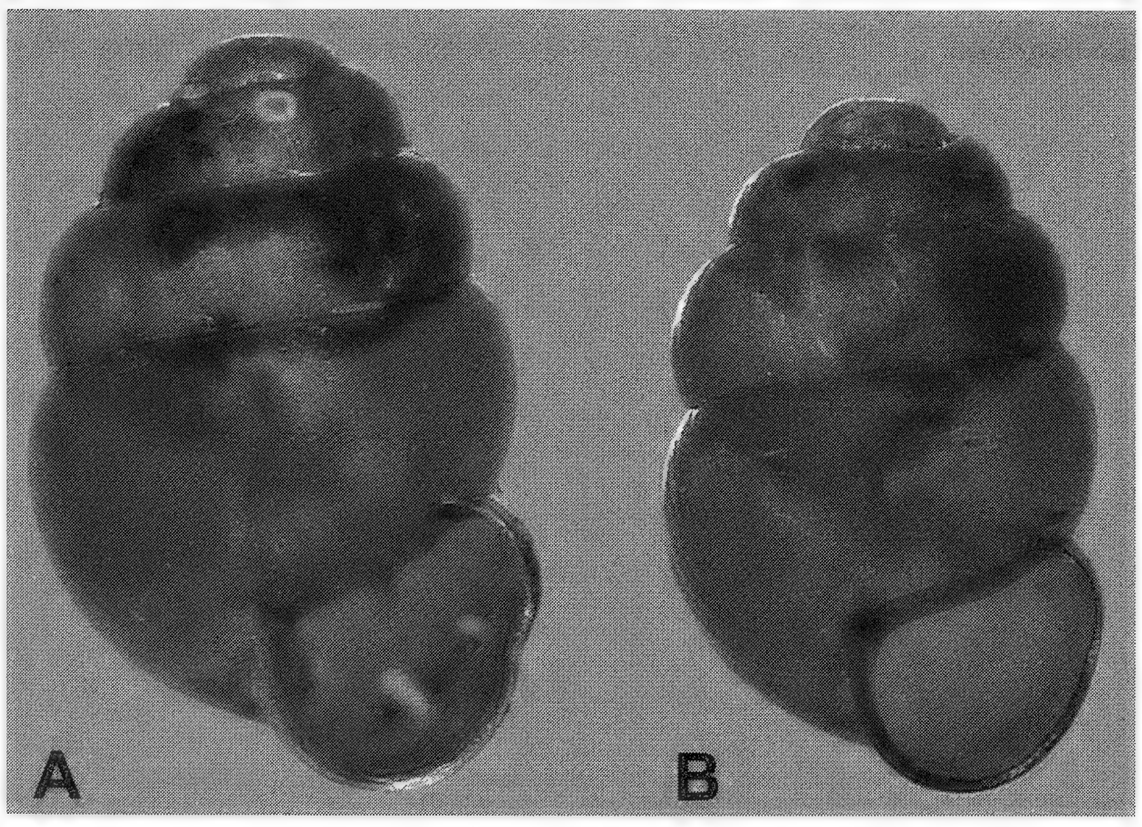


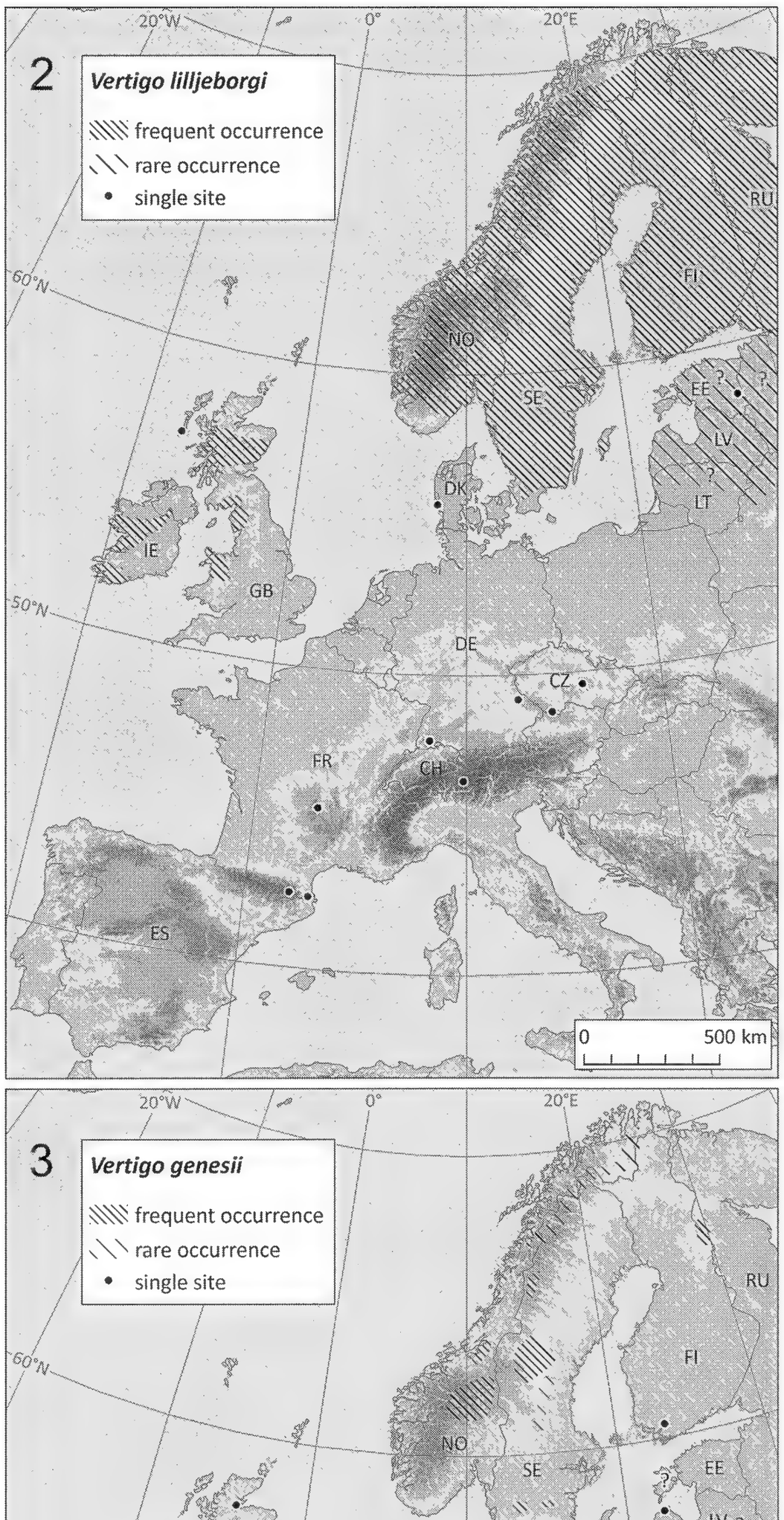
Figure 1. A, *Vertigo lilljeborgi* (shell height 2.02 mm, width 1.39 mm) collected in Mauntschas mire (Upper Engadine valley, SE Swiss Alps). **B,** *Vertigo genesii* (shell height 1.84 mm, width 1.12 mm) collected in an alkaline fen near the village of La Forclaz (Grande Eau valley, W Swiss Alps).

Vertigo lilljeborgi

Geographical range of *Vertigo lilljeborgi* is mainly European, with recently discovered isolated occurrences in Central Asia (Meng 2008, 2009). European distribution of the species is concentrated to Scandinavia, northern part of Great Britain and western Ireland (Kevan and Waterson 1933, Kerney *et al.* 1983, Kerney 1999, von Proschwitz 2003). Its occurrence elsewhere in Europe is represented by a few isolated sites, as summarized in Fig. 2.

Vertigo lilljeborgi is considered to be a typical wetland species, closely associated with open mesotrophic marshes and fens, often of low calcium content (Pokryszko 1990, von Proschwitz 2003). Although it avoids the most acidic sites, such as transitional mires and ombrothrophic bogs, it seems to favor much more acidic conditions than the vast majority of the European land snail fauna (Nekola 2010). The species is strongly hygrophilous, thus it often inhabits swamps at the margins of rivers and lakes, which are subjected to natural over-flooding (Kerney 1999). In central Europe the species usually inhabits wet *Carex* marshes and fens nearby the mountain lakes of glacial origin (*e.g.*, Gerber 1987, von Proschwitz 2004, Lecaplain 2012). According to our earlier study of Scandinavian fens, it most frequently inhabits strongly waterlogged fens moderately rich in calcium, with the occurrence of brown mosses and calcitolerant *Sphagnum* species (Schenková *et al.* unpubl. data). It is worth noticing that whereas in Europe the ecological affinity to acidic sites appears to be very uncommon among land snails, in North America a high proportion of species (largely represented by the genus *Vertigo*) significantly favor acidic sites (Nekola 2010).

Vertigo lilljeborgi was newly discovered in Mauntschas mire near the town of St. Moritz in the Upper Engadine valley (SE Swiss Alps). Mauntschas is a subalpine mire complex nearby the Lake St. Moritz, comprising of ombrothrophic *Sphagnum* bogs, mountain-pine bogs, poor fens and rich fens supplied with spring and runoff water of higher calcium content. The area supporting population of *V. lilljeborgi* belonged to the most mineral-rich spots within the whole area of Mauntschas mire, harboring altogether six mollusc species (Table 1). Relatively high values of water conductivity and water pH (123 μS cm^{-1} and 7.1, respectively) as well as the occurrence of calcicole plant species (*e.g.*, *Carex rostrata*, *Hamatocaulis vernicosus*, *Molinia caerulea*, and *Scorpidium scorpioides*) clearly indicate the impact of mineral rich groundwater on fen surface water chemistry. However, calcitolerant *Sphagna* such as *Sphagnum subsecundum* and *S. warnstorfii*



were also present in the sampling plot. The discovery of *V. lilljeborgi* in Mauntschas mire represents the first record of the species in the Alps, although its occurrence in the mountain range has apparently been anticipated among the European malacologists. Gerber (1987) stated the absence of *V. lilljeborgi* in the Alps as remarkable, while Hausdorf and Hennig (2003) mentioned *V. lilljeborgi* as the only glacial relict land snail, which does not occur in the Alps.

Vertigo genesii

Similarly to *Vertigo lilljeborgi*, geographical range of *V. genesii* is mainly European, with recently discovered isolated occurrences in Central Asia (*e.g.*, Pokryszko and Horsák 2007, Meng 2008, Horsák *et al.* 2010). In Europe, *V. genesii* occurs mainly in Scandinavia, with a scattered distribution across the calcareous parts of the Scandinavian mountain range and calcareous regions in Sweden (von Proschwitz 2003). Rare and isolated occurrences were also documented from the Baltic countries, Great Britain and the Alps, as shown in Fig. 3. Evidences from Poland and Germany are of a doubtful character and were not reliably confirmed up to date (Cameron *et al.* 2003). An attempt to reveal the present distribution of *V. genesii* is mainly complicated by the fact that some older records of *V. genesii* might refer to its sibling species, *V. geyeri* (Kerney *et al.* 1983). *Vertigo geyeri* represents a rare relict from wet glacial periods in central Europe (Ložek 1992) and both ecologically and morphologically resembles *V. genesii* (Kiss and Kopf 2010a). At some sites both species can even co-occur as shown by our record nearby Scuol (E Swiss Alps; 46°46′38″N, 10°16′56″E). Thus, these two species were often confused with each other or not clearly

Figure 2. Present distribution of *Vertigo lilljeborgi* in Europe. The map was constructed based on the records of the species from: **CH,** Switzerland (Schenková and Horsák present work); **CZ,** Czech Republic (Hlaváč unpubl. data, Horsák and Schenková unpubl. data); **DE,** Germany (Hässlein 1964, Gerber 1987); **DK,** Denmark (von Proschwitz 2003); **EE,** Estonia (Remm unpubl. data); **ES,** Spain (von Proschwitz 2004); **FI,** Finland (Kerney *et al.* 1983); **FR,** France (von Proschwitz 2004, Lecaplain 2012); **GB,** Great Britain (Kerney 1999); **IE,** Ireland (Kerney 1999); **LT,** Lithuania (Bank 2011); **LV,** Latvia (Rudzīte *et al.* 2010); **NO,** Norway (von Proschwitz 2003); **RU,** Russia (Kerney *et al.* 1983); and **SE,** Sweden (von Proschwitz 2003).

Figure 3. Present distribution of *Vertigo genesii* in Europe. The map was constructed based on the records of the species from: **AT,** Austria (Kiss and Kopf 2010b); **CH,** Switzerland (http://lepus.unine.ch/carto, Schenková and Horsák present work); **EE,** Estonia (Mänd *et al.* 2002); **FI,** Finland (Valovirta 2003); **GB,** Great Britain (Joint Nature Conservation Committee 2007); **IT,** Italy (Kiss and Kopf 2010a); **LT,** Lithuania (Šatkauskienė 2001); **LV,** Latvia (Pilāte 2000, Rudzīte *et al.* 2010); **NO,** Norway (von Proschwitz 2003); **RU,** Russia (Valovirta 2003); and **SE,** Sweden (von Proschwitz 2003).

Table 1. List of species co-occurring at the newly found sites of *Vertigo lilljeborgi* (site 1) and *V. genesii* in the Swiss Alps (sites 2, 3 and 4).

	Live specimens/empty shells at sites:			
Species	**1**	**2**	**3**	**4**
Aegopinella pura (Alder, 1830)	–	–	–	5/8
Arianta arbustorum (Linnaeus, 1758)	–	–	–	3/19
Carychium minimum O.F. Müller, 1774	–	11/8	28/9	23/69
Carychium tridentatum (Risso, 1826)	–	–	–	8/50
Cochlicopa lubrica (O.F. Müller, 1774)	–	–	–	20/42
Columella columella (Martens, 1830)	–	153/72	–	–
Columella edentula (Draparnaud, 1805)	–	2/0	4/0	0/6
Deroceras agreste (Linnaeus, 1758)	–	1/0	–	1/0
Eucobresia diaphana (Draparnaud, 1805)	–	0/1	–	3/29
Euconulus fulvus (O.F. Müller, 1774)	–	1/2	1/1	0/35
Galba truncatula (O.F. Müller, 1774)	14/10	17/11	0/5	1/1
Perpolita hammonis (Ström, 1765)	2/0	–	1/0	1/2
Perpolita petronella (L. Pfeiffer, 1853)	–	–	–	2/6
Pisidium casertanum (Poli, 1791)	1/0	18/3	–	–
Platyla polita (Hartmann, 1840)	–	–	–	10/9
Punctum pygmaeum (Draparnaud, 1801)	–	0/2	1/1	20/72
Succinea putris (Linnaeus, 1758)	0/5	–	–	–
Vertigo genesii (Gredler, 1856)	–	4/0	40/4	30/75
Vertigo lilljeborgi (Westerlund, 1871)	6/1	–	–	–
Vertigo pygmaea (Draparnaud, 1801)	–	–	–	2/0
Vertigo substriata (Jeffreys, 1833)	6/1	–	2/0	1/31
Vitrea crystallina (O.F. Müller, 1774)	–	2/0	–	–
Vitrina pellucida (O.F. Müller, 1774)	–	–	–	0/1

Site 1: A mineral-rich *Sphagnum*-fen near the town of St. Moritz (Mauntschas mire, SE Swiss Alps), 46°29′21″N, 09°51′08″E, 1828 m a.s.l. **Site 2:** A brown-moss fen near the village of La Forclaz (W Swiss Alps), 46°20′12″N, 07°06′16″E, 1690 m a.s.l. **Site 3:** A brown-moss fen near the village of La Forclaz (W Swiss Alps), 46°20′17″N, 07°06′17″E, 1705 m a.s.l. **Site 4:** A calcareous fen near the village of La Forclaz (W Swiss Alps), 46°20′48″N, 07°04′39″E, 1355 m a.s.l.

distinguished in the older literature. However, in our surveys of fen mollusc assemblages in the Western Carpathians (*e.g.*, Horsák and Cernohorsky 2008), Polish lowlands (Schenková *et al.* 2012) and Bohemian-Moravian Highlands (Horsák and Schenková unpubl. data) we documented many new occurrences of *V. geyeri*, but not a single site of *V. genesii*, despite a high number of ecologically suitable habitats explored.

Vertigo genesii is known to be a stenotopic calciphile species, closely linked to open calcareous fens, spring-fed flushes and seepages in alpine or subalpine zones (Killeen 2003, von Proschwitz 2003). However, the species seems to tolerate slightly less calcareous conditions as well, because in Scandinavia it frequently inhabits fens with calcitolerant peat mosses of *Sphagno warnstorfii-Tomenthypnion* alliance (Schenková *et al.* unpubl. data). In Switzerland it can also be found in very wet rocky subalpine meadows on calcium rich bedrock (Turner *et al.* 1998), while in southern Finland it rarely inhabits partly wooded mires with *Alnus* as well (Valovirta 2003).

Regarding the distribution of *Vertigo genesii* in Switzerland, it has been documented from the Rhaetian Alps in the eastern part of the country (Turner *et al.* 1998). The number of newly found sites in this territory still increases (http://lepus.unine.ch/carto), yet the species has never been observed in the western Alps until now. We found three new sites of *V. genesii* in the vicinity of the village La Forclaz (municipality Ormont-Dessous, Grande Eau valley, W Swiss Alps). Following Hájek *et al.* (2006), two sites could be characterized as extremely rich brown-moss fens, while the third site was a calcareous tufa-forming fen with a strong calcium carbonate precipitation. Complete lists of mollusc species found to co-occur with *V. genesii* are shown in Table 1. All sites were very wet subalpine fens, covered with an almost continuous layer of brown mosses (*e.g.*, *Campylium stellatum*, *Cratoneuron commutatum* and *Drepanocladus cossonii*). Other fen types situated in the surroundings of La Forclaz (*i.e.*, poor *Sphagnum* fen, rich fen with calcitolerant *Sphagna*, seasonally overflooded alkaline fen and calcareous

fen meadow) were explored as well, but *V. genesii* did not occur at any of them.

Conservation remarks

Vertigo genesii and *V. lilljeborgi* are species of very narrow and clearly defined ecological preferences. *Vertigo genesii* is rare and threatened throughout its whole European distribution range, and was, therefore, included among four *Vertigo* species protected under Annex II of the European Union's Habitat Directive (92/43/EEC). In the Red List of molluscs in Switzerland, *V. genesii* is listed in the category "endangered" (Rüetschi *et al.* 2012) and *V. lilljeborgi* should be certainly included as well, because its occurrence in the territory appears to be even more restricted than that of *V. genesii*, at least according to the current knowledge.

Previous studies showed that changes in the water regime and large groundwater level fluctuations are among the most dangerous threats for mollusc fen specialists (*e.g.*, Vavrová *et al.* 2009, Schenková *et al.* 2012), as well as nutrient enrichment (*e.g.*, Pauli *et al.* 2002) and cessation of traditional management practices (Skeffington *et al.* 2006). For the long-term maintenance of viable populations of both studied *Vertigo* species it is crucial to prevent any negative influences mentioned above and to apply suitable site management practices if necessary. Special attention should be paid not only to sites which are known to hold the species, but to all potentially suitable fen habitats as well.

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RESEARCH NOTE

Diet breadth of the northern moonsnail (*Lunatia heros*) on the northwestern Atlantic coast (Naticidae)

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Abstract: *Lunatia heros* (Say, 1822) is a common predatory gastropod in soft-sediment marine environments along the northwestern Atlantic coast. While recognized as a major predator of several commercially important molluscs, little is known about the diet breadth of *L. heros* and the potential of this species to exploit a broader range of molluscan taxa. Here, using a forensic approach based on beach-collected shells, we document prey species drilled by *L. heros* in eastern Cape Breton, Nova Scotia, Canada, and compare our findings to those reported in the literature for this species. Our results indicate that *L. heros* consumes a wider range of prey species than the fifteen currently reflected in the literature. In beach surveys, representatives of twenty of the twenty-eight molluscan species collected were found with beveled boreholes, nine of which were previously unreported as prey items of *L. heros*. Our findings thus confirm the generalist feeding tendencies of this species and increase the number of recorded prey taxa drilled by *L. heros* from fifteen to twenty-four. Further studies of the diet, feeding behavior, and foraging ecology of *L. heros* should ultimately lead to a more comprehensive understanding of this predator and its role in benthic soft-sediment marine environments of the northwestern Atlantic.

Key words: predation, gastropod, marine, soft-sediment, boreholes

The northern moonsnail, *Lunatia heros* (Say, 1822)[1], is a common inhabitant of nearshore soft-sediment marine habitats of the northwestern Atlantic, ranging from Labrador to North Carolina (Rosenberg 2009). Along coastlines spanning this range, the predatory habits of this naticid gastropod are evident in the distinctive beveled boreholes left behind in beach-deposited shells of its molluscan prey (Kabat 1990). While recognized as a major consumer of commercially important bivalves such as *Mya arenaria* Linnaeus, 1758, *Macoma balthica* (Linnaeus, 1758), and *Spisula solidissima* (Dillwyn, 1817) (*e.g.*, Commito 1982, Kelley 1991, Weissberger and Grassle 2003), little is known about the diet breadth of *L. heros* and the potential of this species to exploit a broader range of molluscan taxa. As a consequence, the importance of this predator in coastal food webs and its influence in structuring nearshore soft-sediment environments remain poorly understood. Here, using a forensic approach based on beach-collected shells, we document the diet breadth of *L. heros* in eastern Cape Breton, Nova Scotia, Canada, and compare our results to those previously reported in the literature for this species.

Shell collections were conducted through visual surveys along wave-sheltered and wave-exposed shorelines at Port Morien (46°6′55″N, 59°53′10″W) and Dominion (46°13′17″ N 60°2′24″ W) beaches, on the eastern coast of Cape Breton Island, at biweekly to monthly intervals from May to December in 2008 and 2010. At each sampling period and location, a 0.5–1 km stretch of wave-sheltered and wave-exposed beach was examined during low tide by two or more individuals. In initial collections in both years, all visible shells (> 15 mm shell length) were collected, returned to the lab, identified to species, and subsequently examined for evidence of *Lunatia heros* predation; in subsequent collections, only those shells bearing obvious boreholes were retained. Smaller and more delicate shells potentially overlooked in visual surveys were also targeted by sieving defined patches of sediment (0.35 m x 0.35 m; 2 mm mesh sieve) from areas rich in shell hash at each location. These samples were later examined to identify shell remains and isolate those specimens bearing moonsnail boreholes.

Lunatia heros was assumed to be responsible for all prey taxa with countersunk boreholes in these shell collections. This assumption was supported by the fact that this is the only naticid species recorded along the coastal waters of Eastern Cape Breton Island in the Nova Scotia Museum of Natural History (Andrew Hebda pers. comm.) and was the only

[1] Formerly *Euspira heros*; recently reverted to *Lunatia* Gray, 1847 (Torigoe and Inaba 2011).

naticid species found in our shell collections. Furthermore, borehole characteristics were consistent with those of a naticid gastropod and were also highly uniform within and across prey taxa, suggesting that these boreholes were the product of a single naticid species.

To determine how our dietary analysis of *Lunatia heros* compared to previous studies, we explored the literature to determine those prey species previously identified as dietary components of *L. heros*. This examination consisted of keyword searches in Web of Science and Google Scholar, cited reference searches focusing on significant research/review papers such as Berg and Porter (1974), Kabat (1990), Kelley and Hansen (2003), and Harper (2006), and examinations of the historical literature cited in such papers.

Our literature survey identified fifteen taxa comprising the diet of *Lunatia heros*, including ten bivalve and five gastropod species from eleven different study locales in Canada and the United States (Table 1). One study (Grey *et al.* 2005) identified three prey species that were not included in this list: predation on *Leukoma* (*Protothaca*) *staminea* (Conrad, 1837) was observed in the laboratory, but this bivalve does not overlap in geographic distribution with *L. heros*; two other species - the extinct bivalves *Dallarca idonea* (Conrad, 1832) and *Bornia mactroides* (Conrad, 1834) - were identified as prey of either *L. heros* or a separate, extinct naticid species in a paleontological assessment of naticid drilling, but the specific predator was not determined. Additionally, Giglioli (1949) reported *L. heros* preying on bar clams (species of *Mactra* Linnaeus, 1767) and mussels (species of *Modiolus* Lamarck, 1799). However, since detailed descriptions of predation were lacking, these prey taxa were excluded from our analysis. Drilled prey species not definitively attributed to *L. heros* versus other co-occurring predator species (*e.g.*, *Neverita duplicata* (Say, 1822) and *Lunatia triseriata* (Say, 1826); Alexander and Dietl 2001) were also excluded. Although *L. heros* has also been observed attacking the waved whelk, *Buccinum undatum* Linnaeus, 1758 (Ganong 1889), we are not aware of any reports of successful predation on this species (*i.e.*, completed boreholes).

Our analyses of shell collections from Dominion and Port Morien beaches, Cape Breton Island, revealed a total of twenty prey taxa, including thirteen bivalve and seven gastropod species (Table 1). Of these, six bivalve species (*Arctica islandica* (Linnaeus, 1767), *Heteranomia squamula* (Linnaeus, 1758), *Pandora gouldiana* Dall, 1886, *Petricolaria pholadiformis* (Lamarck, 1818), *Pitar morrhuanus* (Dall, 1902), and *Tellina carpenteri* Dall, 1900) and three gastropod species (*Crepidula fornicata* (Linnaeus, 1758), *Littorina saxatilis* (Olivi, 1792), and *Nucella lapillus* (Linnaeus, 1758)) appear to be new prey records for *Lunatia heros*. Naticid boreholes have been reported previously in shell collections of *Crepidula fornicata* from New Jersey (Cooke *et al.* 2005), but the specific predators associated with these boreholes were not determined. Photographic documentation of the drilled prey taxa recorded in our study is provided in Figure 1; voucher specimens bearing naticid boreholes have also been retained at Cape Breton University and are available upon request. In total, *Lunatia heros* preyed on twenty of twenty-eight (71.4%) molluscan taxa that were collected at these two sites in Cape Breton. Those hard-shelled molluscan taxa lacking evidence of *L. heros* predation were *Buccinum undatum, Crassostrea virginica* (Gmelin, 1791), *Crepidula plana* Say, 1822, *Littorina obtusata* (Linnaeus, 1758), *Modiolus modiolus* (Linnaeus, 1758), *Neptunea decemcostata* (Say, 1826), *Siliqua costata* (Say, 1822), and *Testudinalia testudinalis* (O.F. Müller, 1776). In combination with records from the literature, our results thus increase the number of reported prey taxa for this species from fifteen to twenty-four and confirm the generalist feeding tendencies of this species (Table 1). Coupled with a high incidence of predation on some of these prey species (Clements and Rawlings in prep.), the broad diet of *L. heros* suggests that this predator has the potential to influence the abundance of a suite of shallow-water marine molluscs and shape the structure of soft-sediment benthic communities in the northwestern Atlantic.

Interestingly, the diet breadth of *Lunatia heros* may be even wider than reported above. Similar to other naticids, *L. heros* can potentially consume some prey species without drilling (Kabat 1990). Consequently, such prey items would not be evident in our analysis based on beach-collected drilled shells. While much remains to be determined about the frequency by which naticids feed without drilling, this tactic may be more evident in prey that are unable to seal their body entirely from predators using their shell (Russell-Hunter and Russell-Hunter 1968) or possibly in prey taxa suffering from poor health (Visaggi 2012). Moreover, reports of naticids scavenging on dead fish and crab carcasses and feeding on elasmobranch egg capsules and polychaetes suggest that their diet often extends beyond molluscan taxa (Thorson 1935, Perry 1940, Ansell 1961, Paine 1963, Huelsken 2011). *Lunatia heros*' diet may also change substantially with age (Clements and Rawlings in prep.). For instance, some naticids consume algae and drill non-molluscan prey such as foraminifera and ostracods as early juveniles and later switch to feeding predominantly on bivalves and gastropods (Reyment 1966, 1967, Page and Pedersen 1998, Culver and Lipps 2003, Reyment and Elewa 2003). A broader study spanning the ontogeny of *L. heros*, in conjunction with field observations of its foraging behavior, will be useful in validating and potentially expanding the breadth of this predator's impact in soft-sediment environments.

Of the twenty prey taxa identified along the shores of Cape Breton, six were considered to be rare prey items given that fewer than five drilled shells of each taxon were collected over our entire sampling period (Table 1; *Ensis directus* (Conrad,

Table 1. A list of hard-shelled molluscan taxa preyed upon by *Lunatia heros* in North America based on our beach surveys (2008/2010) and a survey of the published literature. Species level taxonomy conforms to the World Register of Marine Species (Appeltans *et al.* 2012). Species are listed alphabetically within their respective molluscan classes.

Accepted species name	Common name	**Location**	**Reference**
Bivalvia			
Angulus agilis (Stimpson, 1857)	Northern dwarf-tellin	Beverly, MA, USA	Clarke 1956
Arctica islandica (Linnaeus, 1767)*	Ocean quahog	Cape Breton, NS, CAN	-
Divalinga quadrisulcata (d'Orbigny, 1846)	Cross-hatched lucine	NJ, USA	Alexander and Dietl 2001
Ensis directus (Conrad, 1843)†	Atlantic jackknife	Cape Cod, MA, USA	Russell-Hunter and Russell-Hunter 1968
		Cape Breton, NS, CAN	-
Gemma gemma (Totten, 1834)	Gem clam	Beverly, MA, USA	Clarke 1956
		Cape Breton, NS, CAN	-
Heteranomia squamula (Linnaeus, 1758)*†	Jingle shell	Cape Breton, NS, CAN	-
Lunarca ovalis (Bruguière, 1789)	Blood ark	NJ, USA	Alexander and Dietl 2001
Macoma balthica (Linnaeus, 1758)	Baltic macoma	ME, USA	Commito 1982
		Cape Breton, NS, CAN	-
Mercenaria mercenaria (Linnaeus, 1758)	Northern quahog	NY/NJ, USA	MacKenzie 1977
		Cape Breton, NS, CAN	-
Mya arenaria Linnaeus, 1758	Soft-shell clam	Beverly, MA, USA	Clarke 1956
		St. Andrews, NB, CAN	Medcof and Thurber 1958
		Unrecorded	Berg and Porter 1974
		ME, USA	Commito 1982
		NJ, USA	Dietl and Alexander 1997
		Dartmouth, NS, CAN	Kenchington *et al.* 1998
		ME, USA	Beal 2006
		Cape Breton, NS, CAN	-
Mytilus edulis Linnaeus, 1758	Blue mussel	St. Andrews, NB, CAN	Newcombe 1935
		St. Andrews, NB, CAN	Medcof and Thurber 1958
		Dartmouth, NS, CAN	Kenchington *et al.* 1998
		Cape Breton, NS, CAN	-
Pandora gouldiana Dall, 1886*†	Gould's pandora	Cape Breton, NS, CAN	-
Petricolaria pholadiformis (Lamarck, 1818)*	False angelwing	Cape Breton, NS, CAN	-
Pitar morrhuanus (Dall, 1902)*	False quahog	Cape Breton, NS, CAN	-
Spisula solidissima (Dillwyn, 1817)	Atlantic surfclam	NY, USA	Franz 1977
		NJ, USA	Dietl and Alexander 1997
		NJ, USA	Weissberger and Grassle 2003
		NJ, USA	Quijón *et al.* 2007
		Cape Breton, NS, CAN	-
Tellina carpenteri Dall, 1900*	Carpenter tellin	Cape Breton, NS, CAN	-
Gastropoda			
Crepidula fornicata (Linnaeus, 1758)*†	Slipper limpet	Cape Breton, NS, CAN	-
Ilyanassa obsoleta (Say, 1822)	American mudsnail	Unrecorded	Berg and Porter 1974
		West Falmouth, MA, USA	Stenzler and Atema 1977
		Cape Breton, NS, CAN	-
Ilyanassa trivittata (Say, 1822)	Threeline mudsnail	Beverly, MA, USA	Clarke 1956
		Cape Breton, NS, CAN	-
Littorina littorea (Linnaeus, 1758)	Common periwinkle	Nahant, MA, USA	Pechenik *et al.* 2001
		Cape Breton, NS, CAN	-
Littorina saxatilis (Olivi, 1792)*†	Smooth periwinkle	Cape Breton, NS, CAN	-
Lunatia heros (Say, 1822)	Northern moonsnail	Little Cove Point, MD, USA	Kelley 1991
		NJ, USA	Dietl and Alexander 1995
		Cape Breton, NS, CAN	-
Neverita duplicata (Say, 1822)	Shark eye	Little Cove Point, MD, USA	Kelley 1991
		NJ, USA	Dietl and Alexander 1995
Nucella lapillus (Linnaeus, 1758)*†	Dog whelk	Cape Breton, NS, CAN	-

* Novel prey species of *L. heros* identified in this study

† Taxa identified as rare prey items in the diet of *L. heros*

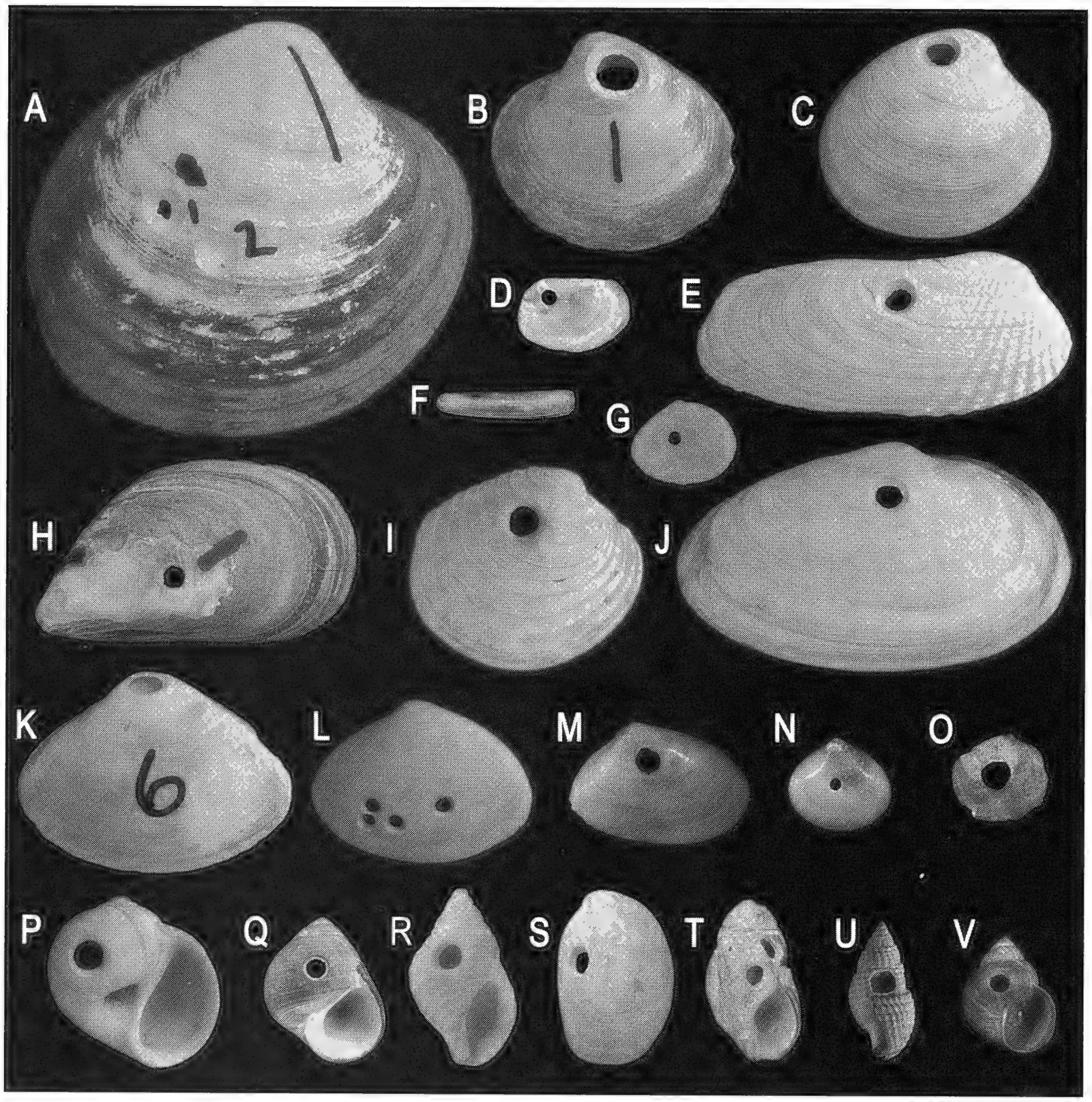

Figure 1. Hard-shelled molluscan prey of *Lunatia heros* from two study locations along the coastline of Cape Breton Island, Nova Scotia. Prey species include: **A,** *Arctica islandica* (two partial boreholes; 65 mm); **B,** *Arctica islandica* (one complete borehole; 35 mm); **C,** *Pitar morrhuanus* (34 mm); **D,** *Pandora gouldiana* (18 mm); **E,** *Petricolaria pholadiformis* (55 mm); **F,** *Ensis directus* (10 mm); **G,** *Macoma balthica* (16 mm); **H,** *Mytilus edulis* (46 mm); **I,** *Mercenaria mercenaria* (26 mm); **J,** *Mya arenaria* (57 mm); **K,** *Spisula solidissima* (single borehole; 40 mm); **L,** *Spisula solidissima* (multiple boreholes; 22 mm); **M,** *Tellina carpenteri* (9 mm) **N,** *Gemma gemma* (3 mm); **O,** *Heteranomia squamula* (4 mm); **P,** *Lunatia heros* (25 mm); **Q,** *Littorina littorea* (23 mm); **R,** *Nucella lapillus* (27 mm); **S,** *Crepidula fornicata* (26 mm); **T,** *Ilyanassa obsoleta* (26 mm); **U,** *Ilyanassa trivittata* (14 mm); and **V,** *Littorina saxatilis* (6 mm). All measurements reflect shell lengths of respective bivalve and gastropod specimens.

1843), *Heteranomia squamula, Pandora gouldiana, Crepidula fornicata, Littorina saxatilis,* and *Nucella lapillus*). In part, these rare instances of predation may reflect low prey densities or habitat differences between predator and prey, such that encounter rates would be expected to be low between *Lunatia heros* and particular prey species. For example, only one drilled shell of *Nucella lapillus* was recorded in all of our beach collections; this whelk is typically associated with rocky habitats rather than the soft-sediment environments of *L. heros*. Alternatively, the rare drilling of taxa such as *Ensis directus* may be a result of increased mobility and subsequent avoidance/escape in this prey species compared to other less mobile prey (Kelley 1991, Kelley and Hansen 1996). Rare instances of predation on species such as *N. lapillus* and *C. fornicata* could also be associated with drilling errors, whereby naticids mistakenly drill empty shells that they encounter (Kabat 1990). Although not overly common, shells bearing more than one complete borehole can be found along Cape Breton shorelines (see Figure 1), which likely reflect the re-drilling of an already drilled prey item or interruptions, repositioning, and/or prey escape during the drilling process (Kitchell *et al.* 1986, Kelley 1991). Ultimately, lab and field based observations of *L. heros*' foraging and drilling behavior are necessary to support the inferences made here based on shell collections alone.

Our results suggest that *Lunatia heros* exploits a broad diet of hard-shelled molluscan prey along the coast of Cape Breton Island. However, given that this study was conducted at two close locations in eastern Canada, further work is necessary to determine the generality of our results to other areas spanning the geographic range of this predator. Despite the generalist feeding tendencies of *L. heros*, this species does feed on some prey species more than others based on the proportion of drilled/non-drilled shells across prey species (Clements and Rawlings in prep.). Additionally, *L. heros* appears to demonstrate gradual ontogenetic shifts in prey selection with growth (Clements 2011). Further studies of the diet of *L. heros*, in combination with analyses of its feeding behavior, foraging ecology, and ontogeny, should ultimately lead to a more comprehensive understanding of this predator and help to illuminate the role that it plays in the benthic soft-sediment marine environments of the northwestern Atlantic.

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RESEARCH NOTE

Caught naked: First report a nudibranch sea slug attacked by a cone snail

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Abstract: A *Conus californicus* Reeve, 1844 is reported killing and attempting to feed on the nudibranch *Triopha catalinae* (Cooper, 1863). This is the first documented case of a predatory cone killing a nudibranch. The impact of cone attacks on nudibranchs populations is unknown, but this note may lead to further research on this topic. Because conotoxins appear to have an effect on the nudibranch nervous system further research on the interaction between conotoxins and nudibranchs could be a source of important discoveries.

Key words: Predation, *Conus californicus*, *Triopha catalinae*, California

Nudibranch sea slugs constitute the most species-rich group of gastropods lacking an adult shell, and exhibit a remarkable degree of morphological diversity. An important reason for the evolutionary success of nudibranchs is the possession of a huge array of defensive mechanisms, including toxicity, venom delivery systems, crypsis, aposematic coloration, mimicry, swimming escape behavior, etc. (Behrens 2005). Nudibranchs possess a wealth of chemical defenses (Cimino and Ghiselin 2009) and empirical data demonstrates that many species are unpalatable (Gosliner 2001, Long and Hay 2006, Haber *et al.* 2010). Additionally, there is significant correlation between conspicuousness and toxicity in nudibranchs and other opisthobranchs (Cortesi and Cheney 2010), suggesting that aposematic colorations have evolved in response to pressure from visual predators. This variety of defensive mechanisms, targeting visual and non-visual predators has served nudibranchs well, and only a handful of enemies have been reported. Rudman (2000) compiled several field reports of attacks on nudibranchs by several animals such as pygnogonids, sea stars, polychaetes, nemerteans, crabs, birds and fish. However, many of these attacks ended up in rejection of the prey. Empirical data and lab experiments have shown that whereas nudibranchs are attacked by different types of predators, very few actually feed on them (Tullrot and Sundberg 1991, Tullrot 1994, Penney 2004). These rejections are an indication that predation on nudibranchs is rare. The best documented cases of nudibranch predation are by pygnogonids (Piel 1991, Arango and Brodie 2003), other opisthobranchs (Paine 1963, Nakano and Hirose 2011), sometimes of the same species (Johnson 1992, Megina and Cervera 2003), and humans (Schrödl 1998).

In this paper, we report and illustrate for the first time a species of nudibranch killed by a cone predatory snail. *Conus californicus* Reeve, 1844 is known to prey on cephalaspidean opisthobranchs (Kohn 1966) and *Conus pennaceus* Born, 1778 has been documented feeding on several groups of opisthobranchs, including cephalaspideans, sea hares and pleurobranchids (Kohn 1959). However, the present note constitutes the first published account of this species (and as far as we know of any species of cone) killing a nudibranch sea slug.

On May 18, 2008 in Hawthorne Reef, Palos Verdes, California (19 m depth, water temperature 11 °C), LB and WM photographed and filmed a *Conus californicus* crawling towards a specimen of *Triopha catalinae* (Cooper, 1863). LB observed the cone approaching the nudibranch and protruding the proboscis to sting the nudibranch on the side of the body. The nudibranch was immediately paralyzed. LB and WM photographed and filmed the cone next to the nudibranch with the siphon of extended toward the nudibranch (Fig. 1). The cone remained next to the nudibranch for at least 10 minutes during which it appeared to attempt to ingest the prey, but engulfing was not observed before the divers discontinued watching.

Conus californicus is an eclectic predator that has been observed feeding on a number of marine animals (Kohn 1966) including fish (Stewart and Gilly 2005), so it is not surprising that they attack nudibranchs. However, such behavior has never been documented. This observation is significant in several respects. Because *Conus californicus* often kills its prey before ingestion (Kohn 1966), even if cones do not ingest the nudibranchs, they could have a significant impact in nudibranch

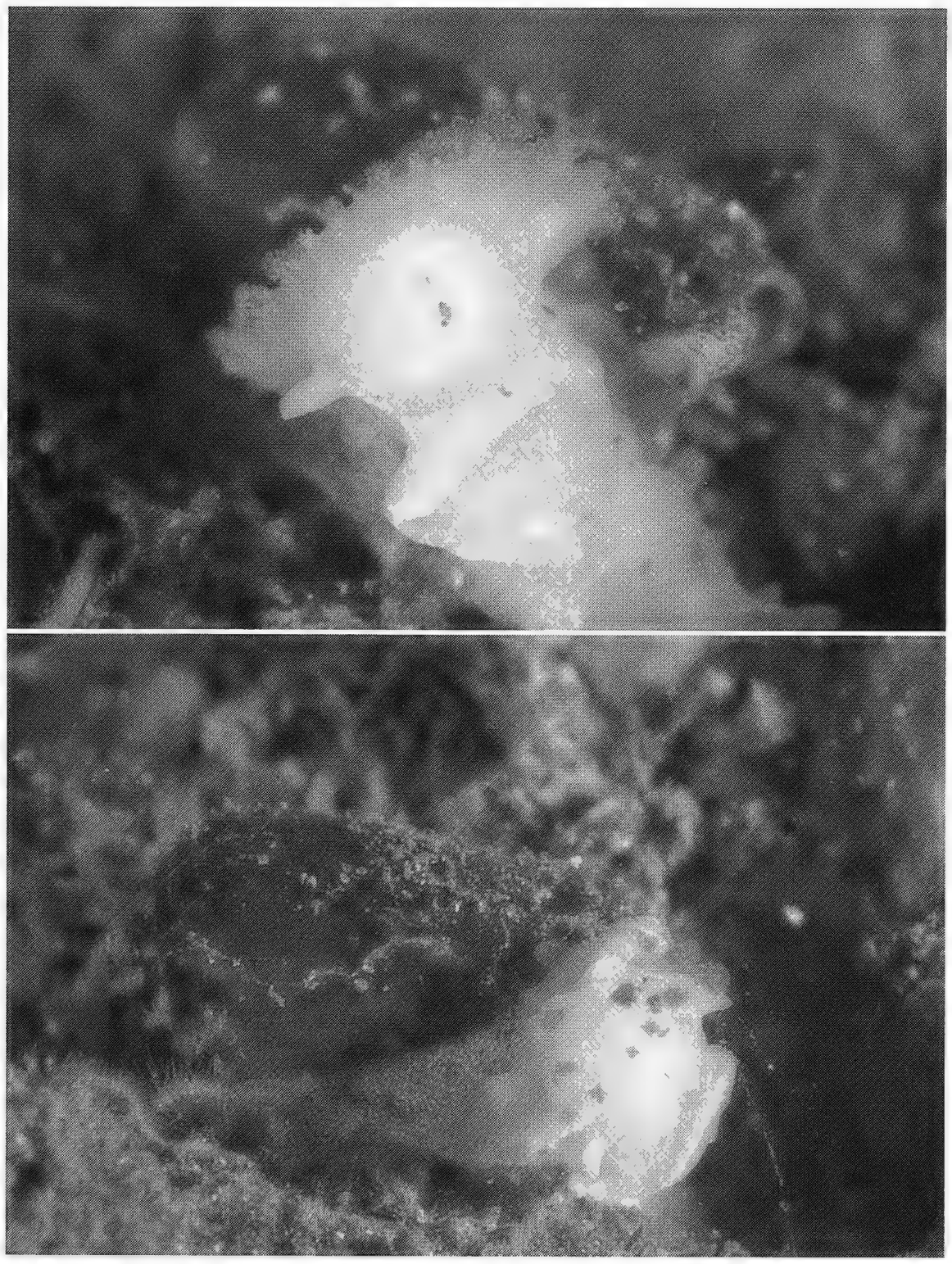

Figure 1. *Conus californicus* "sniffing" a paralyzed *Triopha catalinae* and attempting to engulf the prey.

populations. We hope this note may lead to further research on this topic. Also, because the conotoxins appear to have an effect on the nudibranch nervous system, and some nudibranchs are model organisms for neuroscience (Abraham *et al.* 1972), further research on the interaction between conotoxins and nudibranchs could be a source of important discoveries.

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RESEARCH NOTE

A new species of nudibranch of the genus *Learchis* (Gastropoda: Heterobranchia: Facelinidae) from the tropical western Atlantic Ocean

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Abstract: A new species of aeolid nudibranch is described based on specimens collected in the tropical western Atlantic Ocean, from Cubagua Island, Venezuela. The species belongs to the genus *Learchis* Bergh, 1896. The rhinophoral shape, and body and ceratal coloration are distinct from other previously known species of the genus.

Key words: sea slug, rhinophores, coloration, Venezuela

The number of sea slug species for each of the Atlantic biogeographic areas varies notably with an apparent tendency to increase in diversity from higher to lower latitudes. The classification analysis indicates the existence of a latitudinal gradient in the distribution of the genera. Three main areas of endemism can be distinguished for the Atlantic fauna: south-eastern Atlantic, Magellanic, and Caribbean (García and Bertsch 2009).

The difference in the richness of species in the different Atlantic areas could be due to various factors, such as the intensity and lack of distributional information of some taxonomic and faunistic studies (Gosliner 1987), as well as the fact that different abiotic factors determine the geographic distribution and richness.

The genus *Learchis* Bergh, 1896 consists of two valid species; both of them occurring in Caribbean waters. *Learchis evelinae* (Edmunds and Just, 1983) is reported for Barbados, Martinique, Belize, and Florida (Valdés *et al.* 2006) and *L. poica* (Marcus and Marcus, 1960) is reported for Florida, Mexico, Costa Rica, Jamaica, Aruba, Granada, and Curacao (Valdés *et al.* 2006), and also for the coast of Ghana (Edmunds 1968).

The specimens described here were captured by hand whilst scuba diving at Cubagua Island, in February of 2012. Each individual was observed under a stereoscopic microscope for taking notes of the color patterns and anatomy. Afterwards they were photographed and preserved in 70% ethanol. Complementary to this, illustrations were made of the cerata, the jaw, and the reproductive system using a clear chamber adapted to the stereoscopic microscope.

SPECIES DESCRIPTION

Family Facelinidae Bergh, 1889

Genus *Learchis* Bergh, 1986

***Learchis ignis* sp. nov.**

Material examined

Four specimens measuring 13.1, 9.5, 7.5, and 4.2 mm extended crawling length that were collected in the Bay of Charagato (10°50′26.57″N, 64°9′33.20″W), northeast of Cubagua Island, collected on a shipwreck called *Ferry of Cubagua*, associated with the coral *Tubastrea coccinea* in a depth between 2 and 6 meters. The specimens are deposited in the malacological collection of the Escuela de Ciencias Aplicadas del Mar (number MON005).

External morphology

The body is long and narrow; the smallest specimen examined was 4.2 mm long and the largest was 13.1 mm long. The foot is narrow, and wavy, and it presents a bifurcation in the middle of the body of the animal. The anus is located on the right side of the animal at the base of the second row of ceratas. The proximal half of the rhinophores is smooth and the distal half possesses incomplete and irregular rings. The oral tentacles are twice the length of the rhinophores. The eyes are located in the center immediately behind the base of the rhinophores. The cerata were long and narrow and had a club-shaped form; arranged in two series, they are distributed in six rows starting just after the rhinophores and ending before the tail. Each row has superior and larger cerata and inferior and smaller cerata. The smallest specimen had 28

cerata per series and the largest one had 83, distribution of the cerata for the largest individual is shown in Table 1.

The body is cream in color and it darkens after the second row of cerata because of the coloration of the internal organs. The dorsum has numerous orange speckles and at the base of the tentacles, two triangular orange spots are present. The oral tentacles and the rhinophores are transparent basally and the proximal half is bright yellow. The jaws show through the dorsum as an orange spot. The cerata are mostly pale brown in color except for the end which is orange and the tip is transparent. The internal cnidosac is transparent but it has an orange ring at its base. Finally the tail has a triangular orange streak (Fig. 1a–b). The posture of the animal was flat and circular and in the center the eggs were disorganized (Fig. 2).

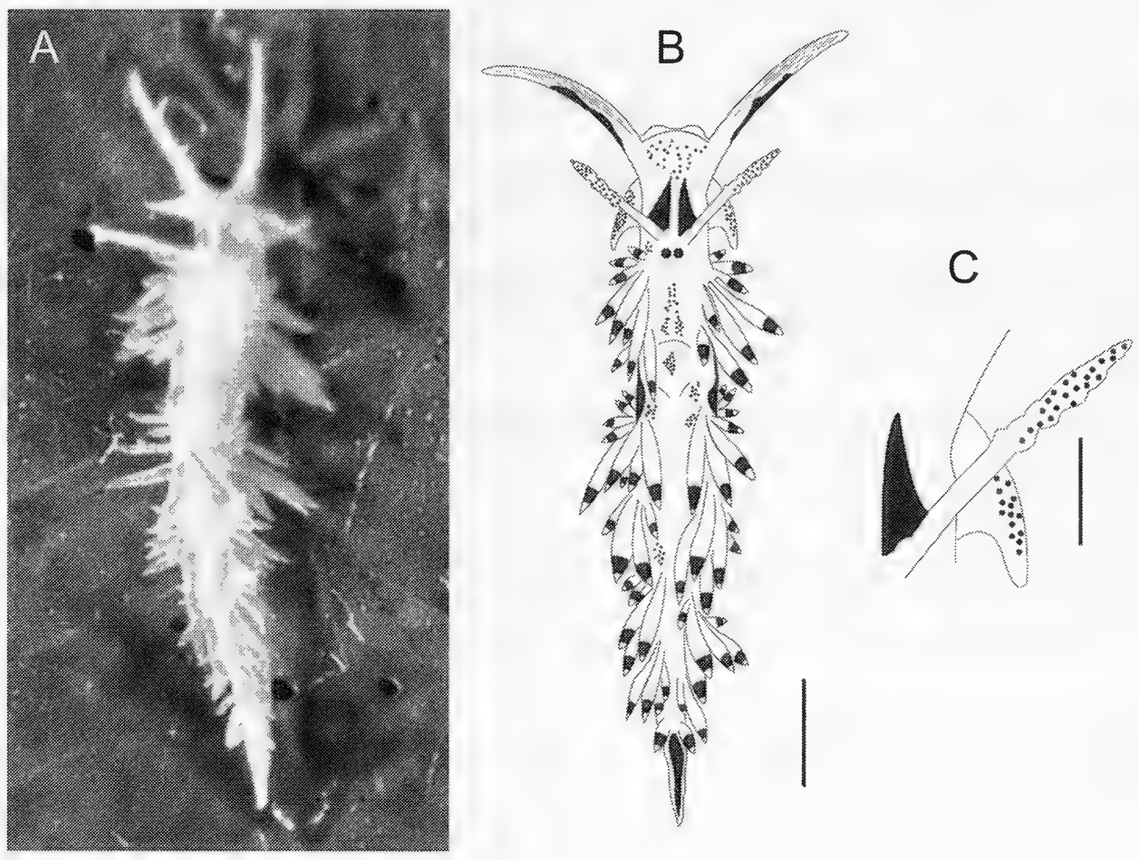

Figure 1. *Learchis ignis* sp. nov. **A,** Photograph of a living specimen from Cubagua Island (taken with a Nikon D90 camera). Specimen length: 13.1 mm; **B,** Drawing of preserved specimen and details of the rhinophora. The shades represent the orange color. Scale bar: 1 mm.

Internal anatomy

The jaw is orange in live animals and ovate, with a masticatory border that consists in one row of 13 regular denticles. The denticles are regular in shape, size and position. The radular formula is 18 x 5.1.5 in the 13.1 mm specimen. The teeth are all smooth and the lateral ones curve slightly inward (Fig. 3). The central tooth is twice as long as the lateral teeth (Fig. 4a–b). The tract of the digestive gland presents a range of color between pale cream and brown. In the reproductive system (Fig. 4c) the ampulla of the hermaphroditic duct is C-shaped and pale orange with the borders more intense, finishing with the hermaphroditic duct. The vas deferens, bursa copulatrix and oviduct were similar to that described by Marcus and Marcus (1960) for *Learchis poica.*

Natural history and geographic range

Individuals were only found on the shipwreck in the Bay of Charagato, Cubagua Island, Venezuela, associated with the coral *Tubastrea coccinea*, between 2–6 m.

Etymology

The specific name *ignis* was given because of the resemblance between the color and shape of the cerata with fire.

Remarks

The placement of this species in *Learchis* (Bergh, 1986) is based upon several features of the genus, as discussed by Marcus and Marcus (1960) such as a jaw with a denticulated masticatory border without a dorsal indentation, the radular tooth with a smooth median cusp flanked by at least three denticles. Also because of the similarity with *L. poica* in the position of the anus, the form of the reproductive system, and the arrangement of the cerata.

Externally, *Learchis ignis* is very different from both the other species of *Learchis*. No other species has yellow on the rhinophores and oral tentacles and also pale brown with orange tipped cerata.

Table 1. Distribution of cerata in the largest specimen of *Learchis ignis* sp. nov.

	Before the digestive gland	After the digestive gland	Total cerata
Right side	13	9, 7, 7, 3, 1	40
Left side	15	9, 8, 6, 4, 1	43

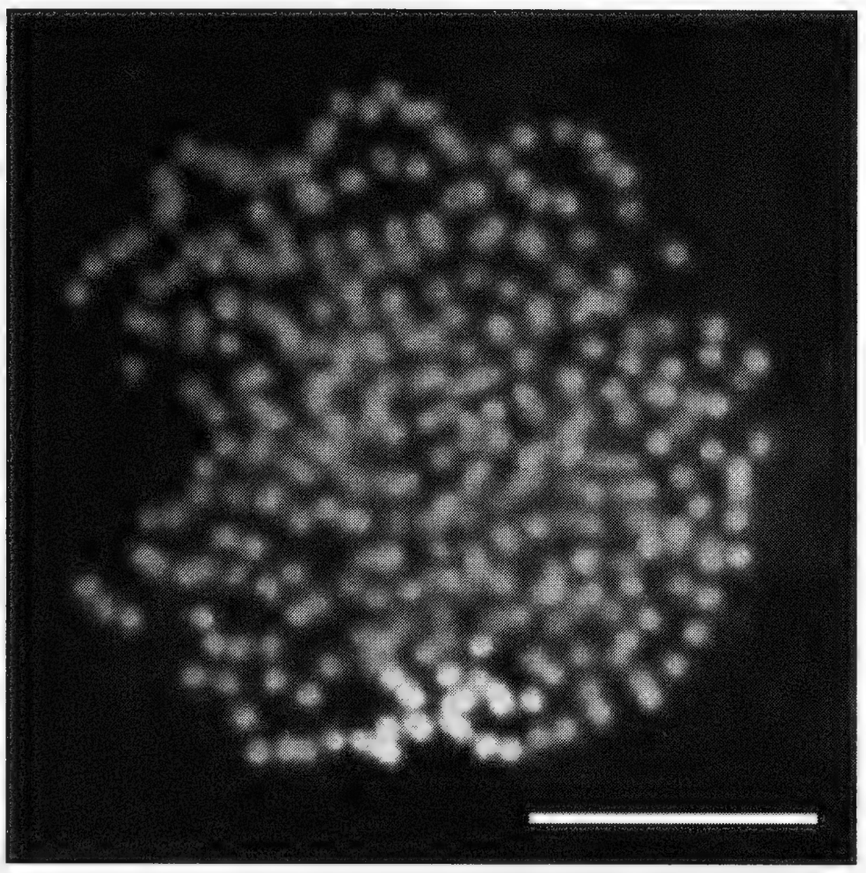

Figure 2. Photograph of the egg mass of *Learchis ignis* sp. nov. from Cubagua Island (taken with a Nikon D90 camera). Scale bar: 1 mm.

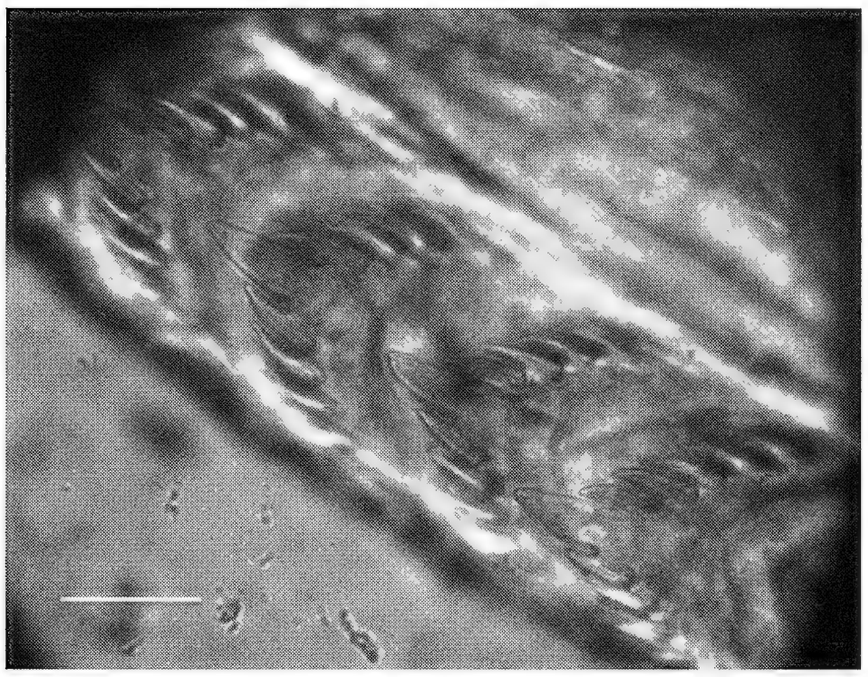

Figure 3. Photograph of the radula of *Learchis ignis* sp. nov. Scale: 1 mm.

The rhinophores have incomplete and irregular rings while in *Learchis poica* the rings are complete and in *L. evelinae* the rhinophores are smooth and lamellate. The number of the cerata per cluster are lower for a similar size specimen than the number described for *L. poica*, also being thinner, as well as the number of radular teeth are lower. The number, shape and size of the denticles on the masticatory border of the jaw in are also different. *L. ignis* oral tentacles are smaller than in *L. evelinae*.

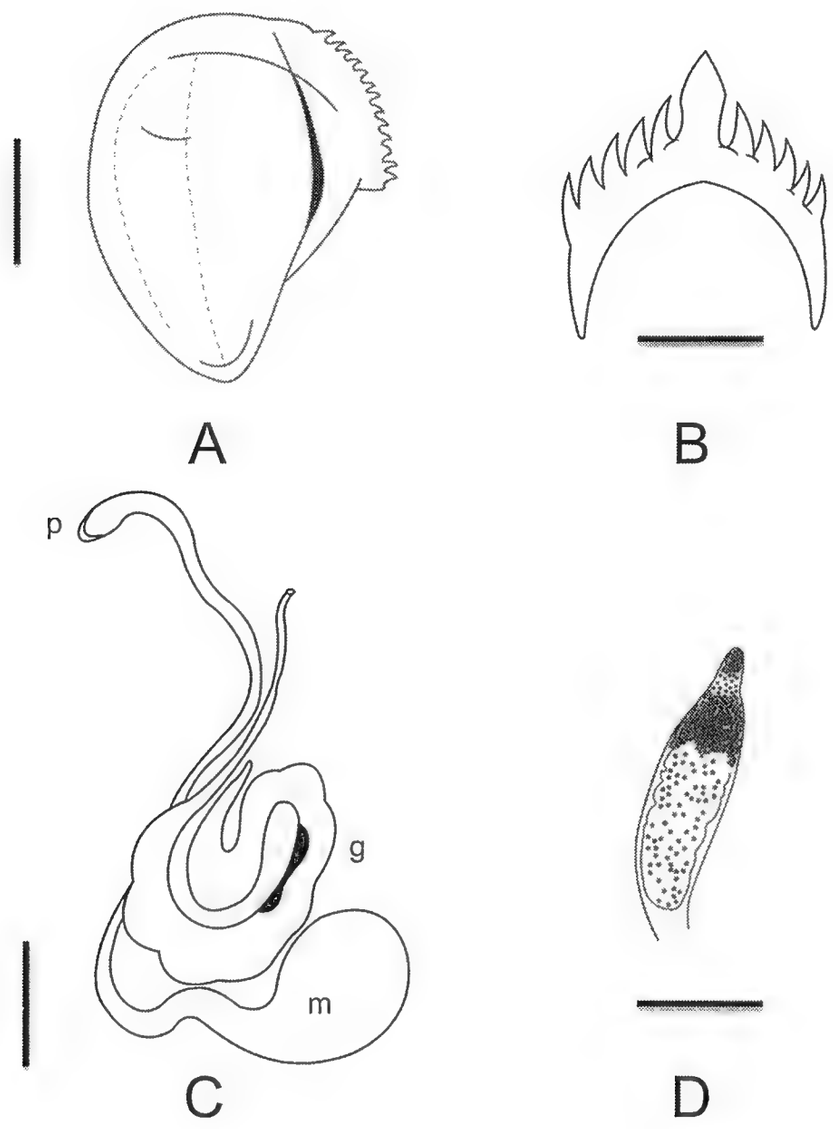

Figure 4. *Learchis ignis* sp. nov. **A,** Jaw; **B,** radula; **C,** reproductive system; and **D,** Ceras. Scale bar: 1 mm. Abbreviations: **g,** female gonad; **m,** male gonad; **p,** penis.

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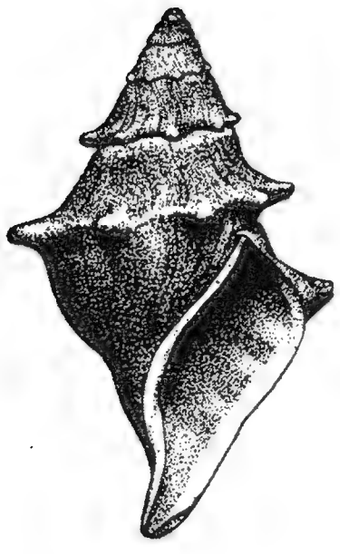

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